METHODS AND COMPOSITIONS FOR THE SPECIFIC COAGULATION OF VASCULATURE

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Disclosed are various compositions and methods for use in achieving specific blood coagulation. This is exemplified by the specific in vivo coagulation of tumor vasculature, causing tumor regression, through the site-specific delivery of a coagulant using a bispecific antibody.

METHODS AND COMPOSITIONS FOR THE SPECIFIC COAGULATION OF VASCULATURE

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DESCRIPTION METHODS AND COMPOSITIONS FOR THE SPECIFIC COAGULATION OF VASCULATURE BACKGROUND OF THE INVENTION 1. Field of the Invention The present invention relates generally to the fields of blood vessels and of coagulation. More particularly, it provides a variety of growth factorbased and immunological reagents, including bispecific antibodies, for use in achieving specific coagulation.

2. Description of the Related Art Advances in the chemotherapy of neoplastic disease have been realized during the last 30 years. This includes some progress in the development of new chemotherapeutic agents and, more particularly, the development of regimens for concurrent administration of drugs. A significant understanding of the neoplastic processes at the cellular and tissue level, and the mechanism of action of basic antineoplastic agents, has also allowed advances in the chemotherapy of a number of neoplastic diseases, including choriocarcinoma, Wilm's tumor, acute leukemia, rhabdomyosarcoma, retinoblastoma, Hodgkin's disease and Burkitt's lymphoma. Despite the advances that have been made in a few tumors, though, many of the most prevalent forms of human cancer still resist effective chemotherapeutic intervention.

A significant underlying problem that must be addressed in any treatment regimen is the concept of "total cell kill." This concept holds that in order to have an effective treatment regimen, whether it be a surgical or chemotherapeutic approach or both, there must be a total cell kill of all so-called "clonogenic" malignant cells, that is, cells that have the ability to grow uncontrolled and replace any tumor mass that might be removed. Due to the ultimate need to develop therapeutic agents and regimens that will achieve a total cell kill, certain types of tumors have been more amenable than others to therapy. For example, the soft tissue tumors (e.g., lymphomas), and tumors of the blood and blood-forming organs (e.g., leukemias) have generally been more responsive to chemotherapeutic therapy than have solid tumors such as carcinomas.

One reason for the susceptibility of soft and bloodbased tumors to chemotherapy is the greater physical accessibility of lymphoma and leukemic cells to chemotherapeutic intervention. Simply put, it is much more difficult for most chemotherapeutic agents to reach all of the cells of a solid tumor mass than it is the soft tumors and blood-based tumors, and therefore much more difficult to achieve a total cell kill. Increasing the dose of chemotherapeutic agents most often results in toxic side effects, which generally limits the effectiveness of conventional anti-tumor agents.

The strategy to develop successful antitumor agents involves the design of agents that will selectively kill tumor cells, while exerting relatively little, if any, untoward effects against normal tissues. This goal has been elusive to achieve, though, in that there are few qualitative differences between neoplastic and normal tissues. Because of this, much research over the years has focused on identifying tumor-specific "marker antigens" that can serve as immunological targets both for chemotherapy and diagnosis. Many tumor-specific, or quasi-tumor-specific ("tumor-associated"), markers have been identified as tumor cell antigens that can be recognized by specific antibodies. Unfortunately, it is generally the case that tumor specific antibodies will not in and of themselves exert sufficient antitumor effects to make them useful in cancer therapy.

More recently, immunotoxins have been employed in an attempt to selectively target cancer cells. Immunotoxins are conjugates of a specific targeting agent, typically a tumor-directed antibody or fragment, with a cytotoxic agent, such as a toxin moiety. The targeting agent is designed to direct the toxin to cells carrying the targeted antigen and to kill such cells. "Second generation" immunotoxins have now been developed, for example, those that employ deglycosylated ricin A chain to prevent entrapment of the immunotoxin by the liver and reduce hepatotoxicity (Blakey et al., 1987a;b), and those with new crosslinkers to endow the immunotoxins with higher in vivo stability (Thorpe et al., 1988).

Immunotoxins have proven effective at treating lymphomas and leukemias in mice (Thorpe et al., 1988; Ghetie et al., 1991; Griffin et al., 1988a;b) and in man (Vitetta et al., 1991). However, lymphoid neoplasias are particularly amenable to immunotoxin therapy because the tumor cells are relatively accessible to blood-borne immunotoxins. Also, it is possible to target normal lymphoid antigens because the normal lymphocytes, which are killed along with the malignant cells during therapy, are rapidly regenerated from progenitors lacking the target antigens.

In contrast with their efficacy in lymphomas, immunotoxins have proved relatively ineffective in the treatment of solid tumors (Weiner et al., 1989; Byers et al., 1989). The principal reason for this is that solid tumors are generally impermeable to antibody-sized molecules: specific uptake values of less than 0.001k of the injected dose/g of tumor are not uncommon in human studies (Sands et al., 1988; Epenetos et al., 1986).

Another significant problem is that antigen-deficient mutants can escape being killed by the immunotoxin and regrow (Thorpe et al., 1988).

Furthermore, antibodies that enter the tumor mass do not distribute evenly for several reasons. Firstly, the dense packing of tumor cells and fibrous tumor stromas present a formidable physical barrier to macromolecular transport and, combined with the absence of lymphatic drainage, create an elevated interstitial pressure in the tumor core which reduces extravasation and fluid convection (Baxter et al., 1991; Jain, 1990). Secondly, the distribution of blood vessels in most tumors is disorganized and heterogeneous, so some tumor cells are separated from extravasating antibody by large diffusion distances (Jain, 1990). Thirdly, all of the antibody entering the tumor may become adsorbed in perivascular regions by the first tumor cells encountered, leaving none to reach tumor cells at more distant sites (Baxter et al., 1991; Kennel et al., 1991).

Thus, it is quite clear that a significant need exists for the development of novel strategies for the treatment of solid tumors. One approach involves the targeting of agents to the vasculature of the tumor, rather than to tumor cells. Solid tumor growth is highly dependent on the vascularization of the tumor and the growth of tumor cells can only be maintained if the supply of oxygen, nutrients and other growth factors and the efflux of metabolic products are satisfactory.

Indeed, it has been observed that many existing therapies may already have, as part of their action, a vascularmediated mechanism of action (Denekamp, 1990).

The present inventors propose that targeting the vasculature will likely deprive the tumor of life sustaining events and result in reduced tumor growth rate or tumor cell death. This approach is contemplated to offer several advantages over direct targeting of tumor cells. Firstly, the target cells are directly accessible to intravenously administered therapeutic agents, permitting rapid localization of a high percentage of the injected dose (Kennel et al., 1991). Secondly, since each capillary provides oxygen and nutrients for thousands of cells in its surrounding 'cord' of tumor, even limited damage to the tumor vasculature could produce an avalanche of tumor cell death (Denekamp, 1990; Denekamp, 1984). Finally, the outgrowth of mutant endothelial cells, lacking a target antigen, is unlikely because they are normal cells.

At the present time, it is generally accepted that for tumor vascular targeting to succeed, antibodies are required that recognize tumor endothelial cells but not those in normal tissues. Although several antibodies have been raised (Duijvestijn et al., 1987; Hagemeier et al., 1986; Bruland et al., 1986; Murray et al., 1989; Schlingemann et al., 1985), none have shown a high degree of specificity. Also, there do not appear to be reports of any particular agents, other than the aforementioned toxins, that show promise as the second agent in a vascular targeted antibody conjugate. Thus, unfortunately, while vascular targeting presents certain theoretical advantages, effective strategies incorporating these advantages have yet to be developed.

SUMMARY OF THE INVENTION The present invention overcomes the limitations of the prior art by providing novel compositions and methods for use in achieving specific coagulation, for example, coagulation in tumor vasculature, with limiting sideeffects. The invention, in a general and overall sense, concerns various novel immunological and growth factorbased bispecific compositions capable of stimulating coagulation in disease-associated vasculature, and methods for their preparation and use.

The invention provides binding ligands that may generally be described as "bispecific binding ligands".

Such ligands comprise a "first binding region" that typically binds to a disease-related target cell, such as a tumor cell, or to a component associated with such a cell; to some component associated with disease-related vasculature, e.g., tumor vasculature; or to a component of, or associated with, disease-associated stroma. The first binding region is operatively associated with or linked to a "coagulating agent", which may be either a coagulation factor itself or may be a second binding region that is capable of binding to a coagulation factor.

The binding ligands of the invention are described as "bispecific" as they are "at least" bispecific, i.e., they comprise, at a minimum, two functionally distinct regions. Compositions and methods using other constructs, such as trispecific and mutlispecific binding ligands, are also included within the scope of the invention. Combined compositions, kits and methods of using the bispecific coagulating ligands described herein in conjunction with other effectors, such as other immunological - and growth-factor-based compositions, antigen-inducing agents, immunostimulants, immunosuppressants, chemotherapeutic drugs, and the like, are also contemplated.

The first binding regions, and any second binding regions, may be antibodies or fragments thereof. As used herein, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgD and IgE. Generally, IgG or IgM are preferred because they are the most common antibodies in the physiological situation and because they are most easily made in a laboratory setting. Monoclonal antibodies (MAbs) are recognized to have certain advantages, e.g., reproducibility and large-scale production, and their use is generally preferred. Engineered antibodies, such as recombinant antibodies and humanized antibodies, also fall within the scope of the invention.

Where antigen binding regions of antibodies are employed as the binding and targeting agent, a complete antibody molecule may be employed. Alternatively, a functional antigen binding region may be used, as exemplified by Fv, scFv (single chain Fv), Fab', Fab, Dab or F(ab')2 fragment of an antibody. The techniques for preparing and using various antibody-based constructs are well known in the art and are further described herein.

The coagulation factor portion of the binding ligands is formed so that it maintains significant functional capacity, i.e., it is in a form so that, when delivered to the target region, it still retains its ability to promote blood coagulation or clotting.

However, in certain embodiments, the coagulation factor portion of the binding ligands will be less active than, for example, the natural counterpart of the coagulant, and the factor will achieve the desired level of activity only upon delivery to the target area. One such example is a vitamin K-dependent coagulation factor that lacks the Gla modification, which will nonetheless achieve significant functional activity upon binding of the first binding region of the bispecific ligand to a membrane environment.

Where a second binding region is used to bind a coagulation factor, it is generally chosen so that it recognizes a site on the coagulation factor that does not significantly impair its ability to induce coagulation.

Likewise, where a coagulation factor is covalently linked to a first binding agent, a site distinct from its functional coagulating site is generally used to join the molecules.

The "first binding region" of the bispecific ligands of the invention may be any component that binds to a designated target site, i.e., a site associated with a tumor region or other disease site in which coagulation is desired. The target molecule, in the case of tumor targeting, will generally be present at a higher concentration in the tumor site than in non-tumor sites.

In certain preferred embodiments, the targeted molecules, whether associated with tumor cells, tumor vascular cells, tumor-associated stroma, or other components, will be restricted to such cells or other tumor-associated entities, however, this is not a requirement of the invention.

In this regard, it should be noted that tumor vasculature is 'prothrombotic' and is predisposed towards coagulation. It is thus contemplated that a targeted coagulant is likely to preferentially coagulate tumor vasculature while not coagulating normal tissue vasculature, even if other normal cells or body components, particularly, the normal endothelial cells or even stroma, express significant levels of the target molecule. This approach is therefore envisioned to be safer for use in humans, e.g., as a means of treating cancer,

than that of targeting a toxin to tumor vasculature.

In certain embodiments, the first binding regions contemplated for use in this invention may be directed to a tumor cell component or to a component associated with a tumor cell. In targeting generally to a tumor cell, it is believed that the first binding ligand will cause the coagulation factor component of the bispecific binding ligand to concentrate on those perivascular tumor cells nearest to the blood vessel and thus trigger coagulation of tumor blood vessels, giving the bispecific binding ligand significant utility.

A first binding region may therefore be a component, such as an antibody or other agent, that binds to a tumor cell. Agents that "bind to a tumor cell" are defined herein as ligands that bind to any accessible component or components of a tumor cell, or that bind to a component that is itself bound to, or otherwise associated with, a tumor cell, as further described herein.

The majority of such tumor-binding ligands are contemplated to be agents, particularly antibodies, that bind to a cell surface tumor antigen or marker. Many such antigens are known, as are a variety of antibodies for use in antigen binding and tumor targeting. The invention thus includes first binding regions, such as antigen binding regions of antibodies, that bind to an identified tumor cell surface antigen, such as those listed in Table I, and first binding regions that preferentially or specifically bind to an intact tumor cell, such as binding to a tumor cell listed in Table II.

Currently preferred examples of tumor cell binding regions are those that comprise an antigen binding region of an antibody that binds to the cell surface tumor antigen p185HER2, milk mucin core protein, TAG-72, Lewis a or carcinoembryonic antigen (CEA). Another group of currently preferred tumor cell binding regions are those that comprise an antigen binding region of an antibody that binds to a tumor-associated antigen that binds to the antibody 9.2.27, OV-TL3, MOv18, B3, KS1/4, 260F9 or D612.

The antibody 9.2.27 binds to high Mr melanoma antigens, OV-TL3 and MOv18 both bind to ovarianassociated antigens, B3 and KS1/4 bind to carcinoma antigens, 260F9 binds to breast carcinoma and D612 binds to colorectal carcinoma. Antigen binding moieties that bind to the same antigen as D612, B3 or KS1/4 are particularly preferred. D612 is described in U.S. Patent 5,183,756, and has ATCC Accession No. HB 9796; B3 is described in U.S. Patent 5,242,813, and has ATCC Accession No. HB 10573; and recombinant and chimeric KS1/4 antibodies are described in U.S. Patent 4,975,369; each incorporated herein by reference.

In tumor cell targeting, where the tumor marker is a component, such as a receptor, for which a biological ligand has been identified, the ligand itself may also be employed as the targeting agent, rather than an antibody.

Active fragments or binding regions of such ligands may also be employed.

First binding regions for use in the invention may also be components that bind to a ligand that is associated with a tumor cell marker. For example, where the tumor antigen in question is a cell-surface receptor, tumor cells in vivo will have the corresponding biological ligand, e.g., hormone, cytokine or growth factor, bound to their surface and available as a target.

This includes both circulating ligands and "paracrine type" ligands that may be generated by the tumor cell and then bound to the cell surface.

The present invention thus further includes first binding regions, such as antibodies and fragments thereof, that bind to a ligand that binds to an identified tumor cell surface antigen, such as those listed in Table I, or that preferentially or specifically binds to one or more intact tumor cells. Additionally, the receptor itself, or preferably an engineered or otherwise soluble form of the receptor or receptor binding domain, could also be employed as the binding region of a bispecific coagulating ligand.

In further embodiments, the first binding region may be a component that binds to a target molecule that is specifically or preferentially expressed in a disease site other than a tumor site. Exemplary target molecules associated with other diseased cells include, for example, PSA associated with Benign Prostatic Hyperplasia (BPH) and FGF associated with proliferative diabetic retinopathy. It is believed that an animal or patient having one of the above diseases would benefit from the specific induction of coagulation in the disease site.

This is the meaning of "diseased cell" in the present context, i.e., it is a cell that is connected with a disease or disorder, which cell expresses, or is otherwise associated with, a targetable component that is present at a higher concentration in the disease sites and cells in comparison to its levels in non-diseased sites and cells. This includes targetable components that are associated with the vasculature in the disease sites.

Exemplary first binding regions for use in targeting and delivering a coagulant to other disease sites include antibodies, such as anti-PSA (BPH), and GF82, GF67 3H3, that bind to FGF. Biological binding ligands, such as FGF, that bind to the relevant receptor, in this case the FGF receptor, may also be used. Antibodies against vascular targets may also be employed, as described below. The targeting of the stroma or endothelial cells provides a powerful means of treating other diseases where the "diseased cell" itself may not be associated with a strong or unique marker antigen.

In further embodiments, the first binding regions of the invention will be components that are capable of binding to a component of disease-associated vasculature, i.e., a region of vasculature in which specific coagulation would be advantageous to the animal or patient. First binding regions capable of binding to a component specifically or preferentially associated with tumor vasculature are currently preferred. "Components of tumor vasculature" include both tumor vasculature endothelial cell surface molecules and any components, such as growth factors, that may be bound to these cell surface receptors or molecules.

Certain preferred binding ligands are antibodies, and fragments thereof, that bind to cell surface receptors and antibodies that bind to the corresponding biological ligands of these receptors. Exemplary antibodies are those that bind to MHC Class II proteins, VEGF/VPF receptors, FGF receptors, TGFP receptors, a TIE (tyrosine kinase-immunoglobulin-epidermal growth factorlike receptor, including TIE-1 and TIE-2), VCAM-1, P-selectin, E-selectin, avss3 integrin, pleiotropin, endosialin and endoglin.

First binding regions that comprise an antigen binding region of an antibody that binds to endoglin are one group of preferred agents. These are exemplified by antibodies and fragments that bind to the same epitope as the monoclonal antibody TEC-4 or the monoclonal antibody TEC-11.

Antigen binding region of antibodies that bind to the VEGF receptor are another group of preferred agents.

These are particularly exemplified by antibodies and fragments that bind to the same epitope as the monoclonal antibody 3E11, 3E7, 5G6, 4D8, 10B10 or TEC-110. Anti VEGF antibodies with binding specificities substantially the same as any one of the antibodies termed 1B4, 4B7, 1B8, 2C9, 7D9, 12D2, 12D7, 12E10, 5E5, 8E5, 5Ell, 7E11, 3F5, 10F3, 1F4, 2F8, 2F9, 2F10, 1G6, 1G11, 3G9, 9G11, 10G9, GV97, GV39, GV97z, GV39y, GV59 or GV14 may also be used. Further suitable anti-VEGF antibodies include 4.6.1., A3.13.1, A4.3.1 and B2.6.2 (Kim et. al., 1992); SBS94.1 (Oncogene Science); G143-264 and G143-856 (Pharmingen).

Further useful antibodies are those that bind to a ligand that binds to a tumor vasculature cell surface receptor. Antibodies that bind to VEGF/VPF, FGF, TGFss, a ligand that binds to a TIE, a tumor-associated fibronectin isoform, scatter factor, hepatocyte growth factor (HGF), platelet factor 4 (PF4), PDGF (including PDGFa and PDGFb) and TIMP (a tissue inhibitor of metalloproteinases, including TIMP-1, TIMP-2 and TIMP-3) are therefore useful in these embodiments, with antibodies that bind to VEGF/VPF, FGF, TGFss, a ligand that binds to a TIE or a tumor-associated fibronectin isoform often being preferred.

In still further embodiments, it is contemplated that markers specific for tumor vasculature may be those that have been first induced, i.e., their expression specifically manipulated by the hand of man, allowing subsequent targeting using a binding ligand; such as an antibody.

Exemplary inducible antigens include those inducible by a cytokine, e.g., IL-1, IL-4, TNF-a, TNF-P or IFN-, as may be released by monocytes, macrophages, mast cells, helper T cells, CD8-positive T-cells, NK cells or even tumor cells. Examples of the induced targets are E-selectin, VCAM-1, ICAM-1, endoglin and MHC Class II antigens. When using MHC Class II induction, the suppression of MHC Class II in normal tissues is generally required, as may be achieved using a cyclosporin, such as Cyclosporin A (CsA), or a functionally equivalent agent.

Further inducible antigens include those inducible by a coagulant, such as by thrombin, Factor IX/IXa, Factor X/Xa, plasmin or a metalloproteinase (matrix metalloproteinase, MMP). Generally, antigens inducible

by thrombin will be used. This group of antigens includes P-selectin, E-selectin, PDGF and ICAM-1, with the induction and targeting of P-selectin and/or E-selectin being generally preferred.

Antibodies that bind to epitopes that are present on ligand-receptor complexes, but absent from both the individual ligand and receptor may also be used. Such antibodies will recognize and bind to a ligand-receptor complex, as presented at the cell surface, but will not bind to the free ligand or uncomplexed receptor. A "ligand-receptor complex", as used herein, therefore refers to the resultant complex produced when a ligand, such as a growth factor, specifically binds to its receptor, such as a growth factor receptor. This is exemplified by the VEGF/VEGF receptor complex.

It is envisioned that such ligand-receptor complexes will likely be present in a significantly higher number on tumor-associated endothelial cells than on non-tumor associated endothelial cells, and may thus be targeted by anti-complex antibodies. Anti-complex antibodies include those antibodies and fragments thereof that bind to the same epitope as the monoclonal antibody 2E5, 3E5 or 4E5.

In further embodiments, the first binding regions contemplated for use in this invention will bind to a component of disease-associated stroma, such as a component of tumor-associated stroma. This includes antigen binding regions of antibodies that bind to basement membrane components, activated platelets and inducible tumor stroma components, especially those inducible by a coagulant, such as thrombin. "Activated platelets" are herein defined as a component of tumor stroma, one reason for which being that they bind to the stroma when activated.

Preferred targetable elements of tumor-associated stroma are currently the tumor-associated fibronectin isoforms. Fibronectin isoforms are ligands that bind to the integrin family of receptors. Tumor-associated fibronectin isoforms are available, e.g., as recognized by the MAb BC-1. This Mab, and others of similar specificity, are therefore preferred agents for use in the present invention. Fibronectin isoforms, although stromal components, bind to endothelial cells and may thus be considered as a targetable vascular endothelial cell-bound ligand in the context of the invention.

Another group of preferred anti-stromal antibodies are those that bind to RIBS, the receptor-induced binding site, on fibrinogen. RIBS is a targetable antigen, the expression of which in stroma is dictated by activated platelets. Antibodies that bind to LIBS, the ligand induced binding site, on activated platelets are also useful.

One further group of useful antibodies are those that bind to tenascin, a large molecular weight extracellular glycoprotein expressed in the stroma of various benign and malignant tumors. Antibodies such as those described by Shrestha et. al. (1994) and 143DB7C8, described by Tuominen & Kallioinen (1994), may thus be used as the binding portions of the coaguligands.

"Components of disease- and tumor-associated stroma" include various cell types, matrix components, effectors and other molecules components that may be considered, by some, to be outside the narrowest definition of "stroma", but are nevertheless targetable entities that are preferentially associated with a disease region, such as a tumor.

Accordingly, the first binding region may be an antibody or ligand that binds to a smooth muscle cell, a pericyte, a fibroblast, a macrophage, an infiltrating lymphocyte or leucocyte. First binding regions may also bind to components of the connective tissue, and include antibodies and ligands that bind to, e.g., fibrin, proteoglycans, glycoproteins, collagens, and anionic polysaccharides such as heparin and heparin-like compounds.

In other preferred embodiments, the vasculature and stroma binding ligands of the invention will be binding regions that are themselves biological ligands, or portions thereof, rather than an antibodies. "Biological ligands" in this sense will be those molecules that bind to or associate with cell surface molecules, such as receptors, that are accessible in the stroma or on vascular cells; as exemplified by cytokines, hormones, growth factors, and the like. Any such growth factor or ligand may be used so long as it binds to the diseaseassociated stroma or vasculature, e.g., to a specific biological receptor present on the surface of a tumor vasculature endothelial cell.

Suitable growth factors for use in these aspects of the invention include, for example, VEGF/VPF (vascular endothelial growth factor/vascular permeability factor), FGF (the fibroblast growth factor family of proteins), TGFP (transforming growth factor B), a ligand that binds to a TIE, a tumor-associated fibronectin isoform,

scatter factor, hepatocyte growth factor (HGF), platelet factor 4 (PF4), PDGF (platelet derived growth factor), TIMP or even IL-8, IL-6 or Factor XIIIa. VEGF/VPF and FGF will often be preferred.

Targeting an endothelial cell-bound component, e.g., a cytokine or growth factor, with a binding ligand construct based on a known receptor is also contemplated.

Generally, where a receptor is used as a targeting component, a truncated or soluble form of the receptor will be employed. In such embodiments, it is particularly preferred that the targeted endothelial cell-bound component be a dimeric ligand, such as VEGF.

This is preferred as one component of the dimer will already be bound to the cell surface receptor in situ, leaving the other component of the dimer available for binding the soluble receptor portion of the bispecific coagulating ligand.

The use of bispecific, or tri- or multi-specific, ligands that include at least one targeting region capable of binding to a component of disease-associated vasculature has the advantage that vascular endothelial cells, and disease-associated agents such as activated platelets, are similar in different diseases, and particularly in different tumors. This phenomenon makes it feasible to treat numerous diseases and types of cancer with one pharmaceutical, rather than having to tailor the agent to each individual disease or specific tumor type.

The compositions and methods of the present invention are thus suitable for use in treating both benign and malignant diseases that have a vascular component. Such vasculature-associated diseases include benign growths, such as BPH, diabetic retinopathy, vascular restenosis, arteriovenous malformations (AVM), meningioma, hemangioma, neovascular glaucoma and psoriasis. Also included within this group are synovitis, dermatitis, endometriosis, angiofibroma, rheumatoid arthritis, atherosclerotic plaques, corneal graft neovascularization, hemophilic joints, hypertrophic scars, osler-weber syndrome, pyogenic granuloma retrolental fibroplasia, scleroderma, trachoma, and vascular adhesions. Each of the above diseases are known to have a common angio-dependent pathology, it is thus contemplated that achieving coagulation in the disease site would prove beneficial.

The bispecific binding ligand-coagulation factor conjugates of the present invention may be conjugates in which the two or more components are covalently linked.

For example, by using a biochemical crosslinker and, preferably, one that has reasonable stability in blood, as exemplified by SMPT. The components may also be linked using the well-known avidin (or streptavidin) and biotin combination. Various cross-linkers, avidin:biotin compositions and combinations, and techniques for preparing conjugates, are well known in the art and are further described herein.

Alternatively, such bispecific coagulating agents may be fusion proteins prepared by molecular biological techniques, i.e., by joining a gene (or cDNA) encoding a binding ligand or region to a gene (or cDNA) encoding a coagulation factor. This is well known in the art and is further described herein. Typically, an expression vector is prepared that comprises, in the same reading frame, a DNA segment encoding the first binding region operatively linked to a DNA segment encoding the coagulation factor and expressing the vector in a recombinant host cell so that it produces the encoded fusion protein.

Coagulation factors for use in the invention may comprise one of the vitamin K-dependent coagulant factors, such as Factor II/IIa, Factor VII/VIIa, Factor IX/IXa or Factor X/Xa. Factor V/Va, VIII/VIIIa, Factor XI/XIa, Factor XII/XIIa and Factor XIII/XIIIa may also be used.

Particular aspects concern the vitamin K-dependent coagulation factors that lack the Gla modification. Such factors may be prepared by expressing a vitamin Kdependent coagulation factor-encoding gene in a procaryotic host cell (which cells are unable to effect the Glu to Gla modification). The factors may also be prepared by making an engineered coagulation factor gene that encodes a vitamin K-dependent coagulation factor lacking the necessary or "corresponding" Glutamic acid residues, and then expressing the engineered gene in virtually any recombinant host cell. Equally, such a coagulation factor may be prepared by treating the vitamin K-dependent coagulation factor protein to remove or alter the corresponding Glutamic acid residues.

Preferred coagulation factors for use in the binding ligands of the invention are Tissue Factor and Tissue

Factor derivatives. One group of useful Tissue Factors are those mutants deficient in the ability to activate Factor VII. A Tissue Factor may be rendered deficient in the ability to activate Factor VII by altering one or more amino acids from the region generally between about position 157 and about position 167 in the amino acid sequence. Exemplary mutants are those wherein Trp at position 158 is changed to Arg; wherein Ser at position 162 is changed to Ala; wherein Gly at position 164 is changed to Ala; and the double mutant wherein Trp at position 158 is changed to Arg and Ser at position 162 is changed to Ala.

Further preferred Tissue Factor derivatives are truncated Tissue Factors, dimeric or even polymeric Tissue Factors and dimeric, or even polymeric, truncated Tissue Factors.

The present invention further provides novel Tissue Factor constructs that comprise a Tissue Factor or derivative operatively linked to at least one other Tissue Factor or derivative. Truncated Tissue Factors are preferred, with truncated Tissue Factors that have been modified to comprise a hydrophobic membrane insertion moiety being particularly preferred.

"A hydrophobic membrane insertion moiety", as defined herein, is one or more units that direct the insertion or functional contact of the Tissue Factor with a membrane. The hydrophobic membrane insertion moieties of the invention are exemplified by stretches of substantially hydrophobic amino acids, such as between about 3 and about 20 hydrophobic amino acids; and also by fatty acids.

The hydrophobic amino acids may be located either at the N- or C-terminus of the truncated Tissue Factor, or appended at another point of the molecule. Where hydrophobic amino acids are used, they may be advantageously incorporated into the molecule by molecular biological techniques. Equally, hydrophobic amino acids or fatty acids may be added to the Tissue Factor using synthetic chemistry techniques.

In the Tissue Factor dimers, trimers and polymers of the present invention, each of the Tissue Factors or derivatives may be operatively linked via, e.g., a disulfide, thioether or peptide bond. In certain embodiments, the Tissue Factor units will be linked via a bond that is substantially stable in plasma, or in the physiological environment in which it is intended for use. This is based upon the inventors' concept that the dimeric form of Tissue Factor may prove to be the most biologically active. However, there is no requirement for a stable linkage as Tissue Factor monomers are known to be active in the methods of the invention.

One or more of the Tissue Factors or truncated Tissue Factors in the dimers and multimers may also be modified to contain a terminal cysteine residue or another moiety that is suitable for linking the Tissue Factor construct to a second agent, such as a binding region.

Tissue Factor monomers, truncated Tissue Factors, and Tissue Factor dimers and multimers that contain a peptide that includes a selectively-cleavable amino acid sequence therefore form another aspect of the invention.

Peptide linkers that include a cleavage site for urokinase, plasmin, Thrombin, Factor IXa, Factor Xa or a metalloproteinase, such as an interstitial collagenase, a gelatinase or a stromelysin, are particularly preferred.

The Tissue Factor monomers, truncated Tissue Factors, Tissue Factor dimers and multimers, and indeed any coagulant, may therefore be linked to a second agent, such as an antibody, an antigen binding region of an antibody, a ligand or a receptor, via a biologicallyreleasable bond. The preference for peptide linkers that include a cleavage site for the above listed proteinases is based on the presence of such proteinases within, e.g., a tumor environment. The delivery of a bispecific agent or ligand to the tumor site is expected to result in cleavage, resulting in the relatively specific release of the coagulation factor.

Particular constructs of the invention are those comprising an operatively linked series of units in the sequence: a cysteine residue, a selectively cleavable peptide linker, a stretch of hydrophobic amino acids, a first truncated Tissue Factor and a second truncated Tissue Factor; or in the sequence: a first cysteine residue, a selectively cleavable peptide linker, a first stretch of hydrophobic amino acids, a first truncated Tissue Factor, a second truncated Tissue Factor and a second stretch of hydrophobic amino acids; wherein each construct may or not be linked to a second agent such as an antibody, ligand or receptor.

Other suitable coagulation factors are Russell's viper venom Factor X activator; platelet-activating

compounds, such as thromboxane A2 and thromboxane A2 synthase; and inhibitors of fibrinolysis, such as a2- antiplasmin.

Also encompassed by the invention are binding ligands in which the coagulation factor is not covalently linked to the conjugate, but is non-covalently bound thereto by means of binding to a second binding region that is operatively linked to the targeting agent of the construct. Suitable "second binding regions" include antigen combining sites of antibodies that have binding specificity for the coagulation factor, including functional portions of antibodies, such as scFv, Fv, Fab', Fab and F(ab')2 fragments.

Binding ligands that contain antibodies, or fragments thereof, directed against the vitamin Kdependent coagulant Factor II/IIa, Factor VII/VIIa, Factor IX/IXa or Factor X/Xa; a vitamin K-dependent coagulation factor that lacks the Gla modification; Tissue Factor, a mutant Tissue Factor, a truncated Tissue Factor, a dimeric Tissue Factor, a polymeric Tissue Factor, a dimeric truncated Tissue Factor; Prekallikein; Factor V/Va, VIII/VIIIa, Factor XI/XIa, Factor XII/XIIa, Factor XIII/XIIIa; Russell's viper venom Factor X activator, thromboxane A2 or a2-antiplasmin are therefore contemplated.

The non-covalently bound coagulating agents may be bound to, or "precomplexed", with a coagulation factor, e.g., so that they may be used to deliver an exogenous coagulation factor to a disease site, e.g., the tumor vasculature, of an animal upon administration. Equally, binding ligands that comprise a second binding region that is specific for a coagulation factor may also be administered to an animal in an "uncomplexed" form and still function to achieve specific coagulation; in which instance, the agent would garner circulating (endogenous) coagulation factor and concentrate it within the disease or tumor site.

In terms of the "coagulation factors" or coagulating agents, these may be endogenous coagulation factors and derivatives thereof, or exogenously added version of such factors, including recombinant versions. Coagulants (in the present "coaguligands") have the distinct advantage over toxins (in immunotoxins) as they will not produce significant adverse side effects upon targeting to a marker that proves to be less than 100k diseaserestricted. Furthermore, the coagulants used will most often be of human origin, and will therefore pose less immunogenicity problems than foreign toxins, such as ricin A chain.

Although not limited to such compositions, important examples of compositions in accordance with this invention are bispecific antibodies, which antibodies comprise a first antigen binding region that binds to a disease cell or component of disease-associated vasculature marker and a second antigen binding region that binds to a coagulation factor. The invention also provides scFv, Fv, Fab', Fab and F(ab')2 fragments of such bispecific antibodies. One currently preferred example of such a bispecific antibody is an antibody comprising one binding site directed against an MHC Class II antigen and another binding site directed against Tissue Factor.

In further embodiments, the present invention provides pharmaceutical compositions of, and therapeutic kits comprising, any or a combination of the above binding ligands and bispecific antibodies in pharmacologically acceptable forms. This includes pharmaceutical compositions and kits where the binding ligand has a first binding region that is covalently linked to a coagulation factor, and also binding ligands in which the first binding region is covalently linked to a second binding region that, in turn, binds to the coagulation factor - whether binding occurs prior to, or subsequent to, administration to an animal.

Pharmaceutical compositions and therapeutic kits that include a combination of bispecific, trispecific or multispecific binding ligands in accordance with the invention are also contemplated. This includes combinations where one binding ligand is directed against a diseased cell or a tumor cell and where another is directed against a vasculature endothelial cell marker or component of disease-associated stroma. Other distinct components may also be included in the compositions and kits of the invention, such as antibodies, immunotoxins, immunoeffectors, chemotherapeutic agents, and the like.

The kits may also include an antigen suppressor, such as a cyclosporin, for use in suppressing antigen expression in endothelial cells of normal tissues; and/or an "inducing agent" for use in inducing diseaseassociated vascular endothelial cells or stroma to express a targetable antigen, such as E-selectin, P-selectin or an MHC Class II antigen. Exemplary inducing agents include T cell clones that bind disease or tumor antigens and that produce IFN-, although it is currently preferred that such clones be isolated from the animal to be treated using the kit.

Preferred inducing agents are bispecific antibodies that bind to disease or tumor cell antigens, or even

stromal components, and to effector cells capable of producing cytokines, coagulants, or other factors, that induce expression of desired target antigens. Currently, one preferred group of bispecific antibodies are those that bind to a tumor antigen and to the activation antigens CD14 or CD16, to stimulate IL-1 production by monocytes, macrophages or mast cells; and those that bind to a tumor antigen and to the activation antigens CD2, CD3 or CD28, and preferably CD28, to stimulate IFNproduction by NK cells or preferably by T cells.

A second preferred group of bispecific antibodies are those that bind to a tumor antigen or to a component of tumor stroma, and to Tissue Factor, a Tissue Factor derivative, prothrombin, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, Factor XI/XIa or Russell's viper venom Factor X activator, to stimulate thrombin production.

Kits comprising such bispecific antibodies as a first "inducing" composition will generally include a second pharmaceutical composition that comprises a binding ligand that comprises a first binding region that binds to P-selectin or E-selectin.

The bispecific ligands of the invention, and other components as desired, may be conveniently aliquoted and packaged, using one or more suitable container means, and the separate containers dispensed in a single package.

Pharmaceutical compositions and kits are further described herein.

Although the present invention has significant clinical utility in the delivery of coagulants and in disease treatment, it also has many in vitro uses. These include, for example, various assays based upon the binding ability of the particular antibody, ligand or receptor, of the bispecific compounds. The bispecific coagulating ligands of invention may thus be employed in standard binding assays and protocols, such as in immunoblots, Western blots, dot blots, RIAs, ELISAs, immunohistochemistry, fluorescent activated cell sorting (FACS), immunoprecipitation, affinity chromatography, and the like, as further described herein.

In still further embodiments, the invention concerns methods for delivering a coagulant to disease-associated vasculature, as may be used to treat diseases such as diabetic retinopathy, vascular restenosis, AVM, hemangioma, neovascular glaucoma, psoriasis and rheumatoid arthritis, and tumors that have a vascularized tumor component. Such methods generally comprise administering to an animal, including a human subject, with a disease that has a vascular component, a pharmaceutical composition comprising at least one bispecific binding ligand in. accordance with those described above.

The compositions are administered in amounts and by routes effective to promote blood coagulation in the vasculature of the disease site, e.g., a solid tumor.

Effective doses will be known to those of skill in the art in light of the present disclosure, such as the information in the Preferred Embodiments and Detailed Examples. Parenteral administration will often be suitable, as will other methods, such as, e.g., injection into a vascularized tumor site.

The methods of the invention provide for the delivery of exogenous coagulation factors, by means of both administering a binding ligand that comprises a covalently-bound coagulation factor and by means of administering a binding ligand that comprises a non-covalently bound coagulation factor that is complexed to a second binding region of the bispecific ligand or antibody.

Further methods of the invention include those that result in the delivery of an endogenous coagulation factor to disease or tumor vasculature. This is achieved by administering to the animal or patient a binding ligand that comprises a second binding region that binds to endogenous coagulation factor and concentrates the factor at the disease-associated or tumor vasculature.

In yet still further methodological embodiments, it is contemplated that markers of tumor vasculature or stroma may be specifically induced and then targeted using a binding ligand, such as an antibody. Exemplary inducible antigens include E-selectin, P-selectin, MMC Class II antigens, VCAM-1, ICAM-1, endoglin, ligands reactive with LAM-1, vascular addressins and other adhesion molecules, with E-selectin and MHC Class II antigens being currently preferred.

When inducing and subsequently targeting MMC Class II proteins, the suppression of MMC Class II in

normal tissues is generally required. MMC Class II suppression may be achieved using a cyclosporin, or a functionally equivalent agent. MHC Class II molecules may then be induced in disease-associated vascular endothelial cells using cyclosporin-independent means, such as by exposing the disease-associated vasculature to an effector cell, generally a Helper T cell or NK cell, of the animal that releases the inducing cytokine IFN-y.

Activated monocytes, macrophages and even mast cells are effector cells capable of producing cytokines (IL-1; TNF-a; TNF-P) that induce E-selectin; whereas Helper T cells, CD8-positive T cells and NK cells are capable of producing IFN-y that induces MMC Class II. Activating monocyte/macrophages in the disease site to produce IL-1, or activating disease-associated Helper T cells or NK cells to produce IFN- , may be achieved by administering to the animal an activating antibody that binds to an effector cell surface activating antigen.

Exemplary activating antigens include CD14 and CD16 (FcR for IgE) for monocytes/macrophages; and CD2, CD3 and CD28 for T cells; with CD14 and CD28, respectively, being preferred for use in certain embodiments.

To achieve specific activation and induction, one currently preferred method is to use a bispecific antibody that binds to both an effector cell activating antigen, such as CD14 or CD28, and to a disease or tumor cell antigen. These bispecific antibodies will localize to the disease or tumor site and activate monocyte/macrophages and T cells, respectively. The activated effector cells in the vicinity of the targeted disease or tumor component will produce inducing cytokines, in this case, IL-1 and IFN-, respectively.

MMC Class II suppression in normal tissues may also be achieved by administering to an animal an anti-CD4 antibody; this functions to suppress IFN-y production by T cells of the animal resulting in inhibition of MMC Class II expression. MMC Class II molecules may again be specifically induced in disease-associated vascular endothelial cells by exposing only the disease site to IFN-y. One means by which to achieve this is by administering to the animal an IFN-y-producing T cell clone that binds to an antigen in the disease site. The IFN-y-producing T cells will preferably be infiltrating leukocytes obtained from the disease site of the animal, such as tumor infiltrating leukocytes (TILs) expanded in vitro.

Methods using bispecific antibodies to induce coagulant, such as thrombin, production, only in a local environment, such as in a tumor site, are also provided.

Again, this will generally be achieved by administering to an animal a pharmaceutical composition comprising a bispecific antibody that binds to a tumor cell or a component of tumor stroma and to Tissue Factor, a Tissue Factor derivative, prothrombin, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, Factor XI/XIa or Russell's viper venom Factor X activator. Antibodies that bind to E-selectin or P-selectin are then linked to a coagulation factor or a second binding region that binds to a coagulation factor and are introduced into the bloodstream of an animal.

More conventional combination treatment regimens are also possible where, for example, a tumor coagulating element of this invention is combined with an existing antitumor therapy, such as with radiotherapy or chemotherapy, or through the use of a second immunological reagent, such as an antitumor immunotoxin.

The novel treatment methods for benign diseases can also be combined with other presently used therapies.

BRIEF DESCRIPTION OF TEK DRAWINGS The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Tethering of tTF to A20 cells via B21-2/10H10 bispecific antibody. A20 cells were incubated with varying concentrations of B21-2/10H10 (z), SFR8/10H10 (z) or B21-2/OX7 (0) plus an excess of 125I-tTF for 1 h at 40C in the presence of sodium azide. The number of 125I- tTF associated with the cells was determined as described in Example II.

FIG. 2. Relationship between number of tethered tTF molecules per A20 cell and ability to induce

coagulation of plasma. A20 cells were incubated with varying concentrations of B21-2/10H10 plus an excess of tTF for 1 h at 40C in the presence of sodium azide. The cells were washed, warmed to 370C, calcium and mouse plasma were added and the time for the first fibrin strands to form was recorded (abscissa). An identical study was performed in which the A20 cells were incubated for 1 h at 40C with bispecific antibody plus 125I-tTF and the number of tTF specifically bound to the cells was determined as described in Example II (ordinate). Plasma added to untreated A20 cells (i.e. zero tTF molecules/cell) coagulated in 190 seconds.

FIG. 3A, FIG. 3B, FIG. 3C and FIG. 3D. Time course of vascular thrombosis and tumor necrosis after administration of coaguligand. Groups of 3 mice bearing 0.8 cm diameter C1300 (mum) tumors were given an intravenous injection of a coaguligand composed of 14 yg B21-2/l0H10 and 11 yg tTF. FIG. 3A; Before injection: blood vessels are intact and tumor cells are healthy.

FIG. 3B; 0.5 hours: blood vessels throughout the tumor are thrombosed; tumor cells are healthy. FIG. 3C; 4 hours: dense thrombi are present in all tumor vessels and tumor cells are separating and developing pyknotic nuclei. Erythrocytes are visible in the tumor interstitium. FIG. 3D; 24 hours: advanced tumor necrosis throughout the tumor. Arrows indicate blood vessels.

FIG. 4. Solid tumor regression induced by tumorvasculature directed coaguligand therapy. Nu/nu mice bearing approximately 0.8 cm diameter C1300 (mum) tumors were given two intravenous injections of B21-2/10H10 (14 yg) mixed with tTF (11 yg) spaced 1 week apart (arrows) (C). Mice in control groups received equivalent doses of tTF alone (O), B21-2/10H10 alone (0) or diluent (R).

Other control groups which received equivalent doses of isotype-matched control bispecific antibodies (SFR8/10H10, OX7/10H10 or B21-2/OX7) and tTF had similar tumor responses to those in animals receiving tTF alone.

The number of mice per group was 7 or 8.

FIG. 5. Exemplary antibody-tTF constructs. This FIG.

shows both the conjugates synthesized by the linkage of chemically derivatized antibody to chemically derivatized tTF via a disulfide bond, and also the linkage of various TF or TF dimers to antibodies and fragments thereof.

FIG. 6. Clotting activity of tTF conjugates when bound to A20 cells. A20 cells were incubated with varying concentrations of B21-2/10H10 bispecific + H6[tTF] in a 1:1 molar ratio, premixed for one hour (z), B21-2 antibody-H6 C[tTF] (.), and B21-2 antibody-H6[tTF] (-) for 1 hour at 40C in the presence of sodium azide. The cells were washed, warmed to 37 C, calcium and mouse plasma were added and the time for the first fibrin strands to form was recorded. The results are expressed as clotting time as a k of the clotting time in the absence of tTF.

FIG. 7. Clotting activity of anti-tumor cell tTF conjugates. LS174T cells (W), Widr cells (.) and H460 cells (#), preincubated with TF9-6B4 and TF8-5G9 antibodies, were incubated with varying concentrations of D612 antibody-H6C[tTF] (W), KS1/4 antibody-H6[tTF) (e), and XMMC0791 antibody-H6[tTF] (r) for 1 hour at 40C in the presence of sodium azide. The cells were washed, warmed to 370C, calcium and mouse plasma were added and the time for the first fibrin strands to form was recorded. The results are expressed as clotting time as a of the clotting time in the absence of tTF.

FIG. 8. Gla domains (z-carboxyglutamic acid) of Factor II/IIa, Factor VII/IIa, Factor IX/IXa and Factor X/Xa.

The arrows represent signal peptide and pro-peptide cleavage sites and activating cleavage sites (slanted arrows).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS Although they show great promise in the therapy of lymphomas and leukemias (Lowder et al., 1987; Vitetta et al., 1991), monoclonal antibodies (MAbs) and immunotoxins (ITs) have thus far proved relatively ineffective in clinical trials against carcinomas and other solid tumors (Byers & Baldwin, 1988; Abrams & BR< Oldham, 1985), which account for more than 90k of all cancers in man (Shockley et al., 1991). A principal reason for this is that macromolecules do not readily extravasate into solid tumors (Sands, 1988; Epenetos et al., 1986) and,

once within the tumor mass, fail to distribute evenly due to the presence of tight junctions between tumor cells (Dvorak et al., 1991), fibrous stroma (Baxter et al., 1991), interstitial pressure gradients (Jain, 1990) and binding site barriers (Juweid et al., 1992).

In developing new strategies for treating solid tumors, the methods that involve targeting the vasculature of the tumor, rather than the tumor cells themselves, therefore seem to offer certain advantages.

Inducing a blockade of the blood flow through the tumor, e.g., through tumor vasculature specific fibrin formation, would interfere with the influx and efflux processes in a tumor site, thus resulting in anti-tumor effect. Arresting the blood supply to a tumor may be accomplished through shifting the procoagulantfibrinolytic balance in the tumor-associated vessels in favour of the coagulating processes by specific exposure to coagulating agents.

The present invention provides various means for effecting specific blood coagulation, as exemplified by tumor-specific coagulation. This is achieved using bispecific or mutlispecific binding ligands in which at least one component is an immunological- or growth factor-based targeting component, and at least one other component is provided that is capable of directly, or indirectly, stimulating coagulation.

A. Targetable Disease Sites The compositions and methods provided by this invention are broadly applicable to the treatment of any disease, such as a benign or malignant tumor, having a vascular component. Such vasculature-associated diseases include BPH, diabetic retinopathy, vascular restenosis, arteriovenous malformations (AVM), meningioma, hemangioma, neovascular glaucoma and psoriasis; and also angiofibroma, arthritis, atherosclerotic plaques, corneal graft neovascularization, hemophilic joints, hypertrophic scars, osler-weber syndrome, pyogenic granuloma retrolental fibroplasia, scleroderma, trachoma, vascular adhesions, synovitis, dermatitis and even endometriosis.

Typical vascularized tumors are the solid tumors, particularly carcinomas, which require a vascular component for the provision of oxygen and nutrients.

Exemplary solid tumors that may be treated using the invention include, but are not limited to, carcinomas of the lung, breast, ovary, stomach, pancreas, larynx, esophagus, testes, liver, parotid, biliary tract, colon, rectum, cervix, uterus, endometrium, kidney, bladder, prostate, thyroid, squamous cell carcinomas, adenocarcinomas, small cell carcinomas, melanomas, gliomas, neuroblastomas, and the like.

One binding region of the bispecific agents of the invention will be a component that is capable of delivering the coagulating agent to the tumor region, i.e., capable of localizing within a tumor site, such as those described above. As somewhat wider distribution of the coagulating agent will not be associated with severe side effects, such as is known to occur with a toxin moiety, there is a less stringent requirement imposed on the targeting element of the bispecific ligand. The targeting agent may thus be directed to components of tumor cells; components of tumor vasculature; components that bind to, or are generally associated with, tumor cells; components that bind to, or are generally associated with, tumor vasculature; components of the tumor extracellular matrix or stroma; and even cell types found within the tumor vasculature.

The burden of very stringent targeting, e.g., as imposed when using immunotoxins, is also lessened due to the fact that tumor vasculature is 'prothrombotic' and is predisposed towards coagulation. Therefore, to achieve specific targeting means that coagulation is promoted in the tumor vasculature relative to the vasculature in nontumor sites. Thus, specific targeting is a functional term rather than a purely physical term relating to the biodistribution properties of the targeting agent, and it is not unlikely that useful targets may be not be entirely tumor-restricted, and that targeting ligands which are effective to promote tumor-specific coagulation may nevertheless be found at other sites of the body following administration.

1. Tumor Cell Targets The malignant cells that make up the tumor may be targeted using a bispecific ligand that has a region capable of binding to a relatively specific marker of the tumor cell. In that binding to tumor cells will localize the associated coagulating agent to the tumor, specific coagulation will be achieved. Furthermore, it is expected that this would be a particularly effective means of promoting coagulation as, due to the physical accessibility of perivascular tumor cells, the bispecific agents will likely be concentrated around the tumor cells that are nearest to a blood vessel.

Many so-called "tumor antigens" have been described, any one which could be employed as a target in connection with the present invention. A large number of exemplary solid tumor-associated antigens are

listed herein in Table I. The preparation and use of antibodies against such antigens is well within the skill of the art, and exemplary antibodies are also listed in Table I.

Another means of defining a targetable tumor is in terms of the characteristics of a tumor cell itself, rather than describing the biochemical properties of an antigen expressed by the cell. Accordingly, Table II is provided for the purpose of exemplifying human tumor cell lines that are publically available (from ATCC Catalogue).

The information presented in Table II is by means of an example, and not intended to be limiting either by year or by scope. One may consult the ATCC Catalogue of any subsequent year to identify other appropriate cell lines. Also, if a particular cell type is desired, the means for obtaining such cells, and/or their instantly available source, will be known to those of skill in the particular art. An analysis of the scientific literature will thus readily reveal an appropriate choice of cell for any tumor cell type desired to be targeted.

TABLE I MARKER ANTIGENS OF SOLID TUMORS AND CORRESPONDING MONOCLONAL ANTIBODIES EMI37.1

Tumor	Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
A:	'CA 125'	> 200 kD GP	OC 125	Kabawat et al., 1983; Szymendera, 1986
Gynecological		mucin		
GY				
ovarian		80 Kd GP	OC 133	Masuko et al, Cancer Res., 1984
ovarian		'SGA' 360 Kd GP	OMI	de Krester et al., 1986
ovarian		High Mr mucin	Mo v1	Miotti et al, Cancer Res., 1985
ovarian		High Mr mucin/ glycolipid	Mo v2	Miotti et al, Cancer Res., 1985
ovarian		NS	3C2	Tsuji et al., Cancer Res., 1985
ovarian		NS	4C7	Tsuji et al., Cancer Res., 1985
ovarian		High Mr mucin	ID3	Gangopadhyay et al., 1985
ovarian		High Mr mucin	DU-PAN-2	Lan et al., 1985
GY		7700 Kd GP	F 36/22	Croghan et al., 1984
ovarian		'gp 68' 48 Kd GP	4F7/7A10	Bhattacharya et al., 1984
GY		40, 42kD GP	OV-TL3	Poels et al., 1986
GY		'TAG-72' High Mr mucin	B72.3	Thor et al., 1986

TABLE I continued... EMI38.1

Tumor	Site	Antigen Identity/ Characteristics	Monoclonal Antibodies	Reference
ovarian		300-400 Kd GP	DF3	Kufe et al., 1984
ovarian		60 Kd GP	2C8/2F7	Bhattacharya et al., 1985
GY		105 Kd GP	MF 116	Mattes et al., 1984
ovarian		38-40 kD GP	MOv18	Miotti et al., 1987
GY		'CEA' 180 Kd GP	CEA 11-H5	Wagener et al., 1984
ovarian		CA 19-9 or GICA (1116NS 199)	CA 19-9	Atkinson et al., 1982
ovarian		'PLAP' 67 Kd GP	H17-E2	McDicken et al., 1985
ovarian		72 Kd	791T/36	Perkins et al., 1985
ovarian		69 Kd PLAP	NDOG2	Sunderland et al., 1984
ovarian		unknown Mr PLAP	H317	Johnson et al., 1981
ovarian		p185HER2	4D5, 3H4, 7C2, 6E9, 2C4, 7F3, 2H11, 3E8, 5B8, 7D3, SB8	Shepard et al., 1991
uterus	ovary	HMFG-2	HMFG2	Epenetos et al., 1982
GY		HMFG-2	3.14.A3	Burchell et al., 1983

TABLE I continued... EMI39.1

Tumor Site	Antigen Characteristics	Identity/ Antibodies	Monoclonal Reference
B: BREAST	330-450 Kd GP	DF3	Hayes et al., 1985
NS	NCRC-11	Ellis et al., 1984	
37kD	3C6F9	Mandeville et al., 1987	
NS	MBE6	Teramoto et al., 1982	
NS	CLNH5	Glassy et al., 1983	
47 Kd GP	MAC 40/43	Kjeldsen et al., 1986	
High Mr GP	EMA	Sloane et al., 1981	
High Mr GP	HMFG1 HFMG2	Arklie et al., 1981	
NS	3.15.C3	Arklie et al., 1981	
NS	M3, M8, M24	Foster et al., 1982	
1 (Ma) blood group Ags	M18	Foster et al., 1984	
NS	67-D-11	Rasmussen et al., 1982	
oestrogen receptor	D547Sp, D75P3, H222	Kinsel et al., 1989	
EGF Receptor	Anti-EGF	Sainsbury et al., 1985	
Laminin Receptor	LR-3	Horan Hand et al., 1985	
erb B-2 p185	TA1	Gusterson et al., 1988	
NS	H59	Hendler et al., 1981	

TABLE I continued... EMI40.1

Tumor Site	Antigen Characteristics	Identity/ Antibodies	Monoclonal Reference
126 Kd GP	10 - 3D - 2	Soule et al., 1983	
NS	HmAB1,2	Imam et al., 1984; Schlom et al., 1985	
NS	MBR 1,2,3	Menard et al., 1983	
95 Kd	24.17.1	Thompson et al., 1983	
100 Kd (3E1.2)	24.17.2	Croghan et al., 1983	
NS	F36/22.M7/10	Croghan et al., 1984	
5 24 Kd	C11, G3, H7	Adams et al., 1983	
90 Kd GP	B6.2	Colcher et al., 1981	
CEA & 180 Kd GP	B1.1	Colcher et al., 1983	
colonic & parcreatic muncin similar to Ca 199	Cam 17.1	Imperial Cancer Research Technology MAb listing	
milk mucin core protei	SM3	Imperial Cancer Research Technology Mab listing	
milk mucin core protei	SM4	Imperial Cancer Research Technology Mab listing	
affinity-purified milk mucin	C-Mul (566)	Imperial Cancer Research Technology Mab listing	

TABLE I continued... EMI41.1

Tumor Site	Antigen Characteristics	Identity/ Antibodies	Monoclonal Reference
p185HER2	4D5	3H4, 7C2, 6E9, 2C4, 7F3, 2H11, 3E8, 5B8, 7D3, 5B8	Shepard et al., 1991
CA 125	> 200 Kd GP	OC 125	Kabawat et al., 1985
High Mr mucin/	MO v2	Miotti et al., 1985	

glycolipid
High Mr mucin DU-PAN-2 Lan et al., 1984
'gp48' 48 Kd GP 4F7/7A10 Bhattacharya et al., 1984
300-400 Kd GP DF3 Kufe et al., 1984
'TAG-72' high Mr B72.3 Thor et al., 1986
mucin
'CEA' 180 Kd GP cccccCEA 11 Wagener et al., 1984
'PLAP' 67 Kd GP H17-E2 McDicken et al., 1985
HMFG-2 > 400 Kd GP 3.14.A3 Burchell et al., 1983
NS FO23C5 Riva et al., 1988
C: COLORECTAL TAG-72 High Mr B72.3 Colcher et al., 1987
mucin
GP37 (17-1A) Paul et al., 1986
1083-17-1A
TABLE I continued... EMI42.1

Tumor Site Antigen Identity/ Monoclonal Reference
Characteristics Antibodies
Surface GP CO17-1A LoBuglio et al., 1988
CEA ZCE-025 Patt et al., 1988
CEA AB2 Griffin et al., 1988a
cell surface AG HT-29-15 Cohn et al., 1987
secretory 250-30.6 Leydem et al., 1986
epithelium
surface 44x14 Gallagher et al., 1986
glycoprotein
NS A7 Takahashi et al., 1988
NS GA73.3 Munz et al., 1986
NS 791T/36 Farrans et al., 1982
cell membrane & 28A32 Smith et al., 1987
cytoplasmic Ag
CEA & vindesine 28.19.8 Corvalen, 1987
gp72 X MMCO-791 Byers et al., 1987
high Mr mucin DU-PAN-2 Lan et al., 1985
high Mr mucin ID3 Gangopadhyay et al., 1985
CEA 180 Kd GP CEA 11-H5 Wagener et al., 1984
60 Kd GP 2C8/2F7 Bhattacharya et al., 1985

TABLE I continued... EMI43.1

Tumor Site Antigen Identity/ Monoclonal Reference
Characteristics Antibodies
CA-19-9 (or GICA) CA-19-9 Atkinson et al., 1982
(1116NS 199)
Lewis a PR5C5 Imperial Cancer Research Technology
Mab Listing
Lewis a PR4D2 Imperial Cancer Research Technology
Mab Listing
colonic mucus PR4D1 Imperial Cancer Research Technology
Mab Listing
D: MELANOMA p97a 4.1 Woodbury et al., 1980
p97a 8.2 M17 Brown, et al., 1981a
p97b 96.5 Brown, et al., 1981a
p97c 118.1, Brown, et al., 1981a
133.2,
(113.2)
p97c L1, L10, Brown et al., 1981b
R10 (R19)
p97d I12 Brown et al., 1981b

p97e K5 Brown et al., 1981b
P155 6.1 Loop et al., 1981
GD3 disialogan- R24 Dippold et al., 1980
glioside
TABLE I continued... EMI44.1

Tumor Site Antigen Identity/ Monoclonal Reference
Characteristics Antibodies
p210, p60, p250 5.1 Loop et al., 1981
p280 p440 225.28S Wilson et al., 1981
GP 94, 75, 70 & 465.12S Wilson et al., 1981
25
P240-P250. P450 9.2.27 Reisfeld et al., 1982
100, 77, 75 Kd F11 Chee et al., 1982
94 Kd 376.96S Imai et al., 1982
4 GP chains 465.12S Imai et al., 1982; Wilson et al., 1982
GP 74 15.75 Johnson & Reithmuller, 1982
GP 49 15.95 Johnson & Reithmuller, 1982
230 Kd Mel-14 Carrel et al., 1982
92 Kd Mel.12 Carrel et al., 1982
70 Kd Me3-TB7 Carrel et al., 1:::387, 1982
HMW MAA similar 225.28SD Kantor et al., 1982
to 9.2.27 AG
HMW MAA similar 763.24TS Kantor et al., 1982
to 9.2.27 AG
GP95 similar to 705F6 Stuhlmiller et al., 1982
376.96S 465.12S
GP125 436910 Saxton et al., 1982
TABLE I continued... EMI45.1

Tumor Site Antigen Indentity/ Monoclonal Reference
Characteristics Antibodies
CD41 M148 Imperial Cancer Research Technology
Mab listing
E: high Mr mucin ID3 Gangopadhyay et al., 1985
GASTROINTESTI
NAL
pancreas
stomach
gall bladder, high Mr mucin DU-PAN-2 Lan et al., 1985
pancreas,
stomach
pancreas NS OV-TL3 Poels et al., 1984
pancreas 'TAG-72' high Mr B72.3 Thor et al., 1986
stomach, mucin
oesophagus
stomach 'CEA' 180 Kd GP CEA 11-H5 Wagener et al., 1984
pancreas HMFG-2 > 400 Kd GP 3-14-A3 Burchell et al., 1983
G.I. NS C COLI Lemkin et al., 1984
pancreas, CA 19-9 (or GICA) CA-19-9 Szymendera, 1986
stomach (1116NS 199) and CA50
pancreas CA125 GP OC125 Szymendera, 1986

TABLE I continued... EMI46.1

Tumor Site Antigen Identity/ Monoclonal Reference
Characteristics Antibodies
F: LUNG p185HER2 4D5 3H4, Shepard et al., 1991
7C2, 6E9,

2C4, 7F3,
2H11, 3E8,
non-small
5B8, 7D3,
cell lung
SB8
carcinoma
high Mr mucin/ MO v2 Miotti et al., 1985
glycolipid
'TAG-72' high Mr B72.3 Thor et al., 1986
mucin
high Mr mucin DU-PAN-2 Lan et al., 1985
'CEA' 180 kD GP CEA 11-H5 Wagener et al., 1984
Malignant cytoplasmic MUC 8-22 Stavrou, 1990
Gliomas antigen from
85HG-22 cells
cell surface Ag MUC 2-63 Stavrou, 1990
from 85HG-63
cells
cell surface Ag MUC 2-39 Stavrou, 1990
from 86HG-39
cells

TABLE I continued.. EMI47.1

Tumor Site Antigen Identity/ Monoclonal Reference
Characteristics Antibodies
cell surface Ag MUC 7-39 Stavrou, 1990
from 86HG-39
cells
G: p53 PAb 240 Imperial Cancer Research Technology
MISCELLANEOUS PAb 246 MaB Listing
PAb 1801
small round neural cell ERIC.1 Imperial Cancer Research Technology
cell tumors adhesion molecule MaB Listing
medulloblasto M148 Imperial Cancer Research Technology
ma MaB Listing
neuroblastoma
rhabdomyosarc
oma
neuroblastoma FMH25 Imperial Cancer Research Technology
MaB Listing
renal cancer p155 6.1 Loop et al., 1981
& BR< glioblastomas
bladder & "Ca Antigen" 350- CA1 Ashall et al., 1982
laryngeal 390 kD
cancers
neuroblastoma GD2 3F8. Cheung et al., 1986
Prostate gp48 48 kD GP 4F7/7A10 Bhattacharya et al., 1984
Prostate 60 kD GP 2C8/2F7 Bhattacharya et al., 1985

TABLE I continued... EMI48.1

Tumor Site Antigen Identity/ Monoclonal Reference
Characteristics Antibodies
Thyroid 'CEA' 180 kD GP CEA 11-H5 Wagener et al., 1984
abbreviations: Abs, antibodies; Ags, antigens; EGF, epidermal growth factor; GI, gastrointestinal; GICA, gastrointestinal-associated antigen; GP, glycoprotein; GY, gynecological; HMFG, human milk fat globule;

Kd, kilodaltons; Mabs, monoclonal antibodies; Mr, molecular weight; NS, not specified; PLAP, placental alkaline phosphatase; TAG, tumor-associated glycoprotein; CEA, carcinoembryonic antigen.

footnotes: the CA 19-9 Ag (GICA) is sialosylfucosyllactotetraosylceramide, also termed sialylated Lewis pentaglycosyl ceramide or sialyated lacto-N-fucopentaose II; p97 Ags are believed to be chondroitin sulphate proteoglycan; antigens reactive with Mab 9.2.27 are believed to be sialylated glycoproteins associated with chondroitin sulphate proteoglycan; unless specified, GY can include cancers of the cervix, endocervix, endometrium, fallopian tube, ovary, vagina or mixed Mullerian tumor; unless specified GI can include cancers of the liver, small intestine, spleen, pancreas, stomach and oesophagus.

TABLE II HUMAN TUMOR CELL LINES AND SOURCES EMI49.1

ATTC NUMBER	HTB	CELL LINE	TUMOR TYPE
1	J82		Transitional-cell carcinoma, bladder
2	RT4		Transitional-cell papilloma, bladder
3	ScaBER		Squamous carcinoma, bladder
4	T24		Transitional-cell carcinoma, bladder
5	TCCSUP		Transitional-cell carcinoma, bladder, primary grade Iv
9	5637		Carcinoma, bladder, primary
10	SK-N-MC		Neuroblastoma, metastasis to supra-orbital area
11	SK-N-SH		Neuroblastoma, metastasis to bone marrow
12	SW	1088	Astrocytoma
13	SW	1783	Astrocytoma
14	U-87	MG	Glioblastoma, astrocytoma, grade III
15	U-118	MG	Glioblastoma
16	U-138	MG	Glioblastoma
17	U-373	MG	Glioblastoma, astrocytoma, grade III
18	Y79		Retinoblastoma
19	BT-20		Carcinoma, breast
20	BT-474		Ductal carcinoma, breast
22	MCF7		Breast adenocarcinoma, pleural effusion
23	MDA-MB-134-VI		Breast, ductal carcinoma, pleural effusion
24	MDA-MD-157		Breast medulla, carcinoma, pleural effusion
25	MDA-MB-175-VII		Breast, ductal carcinoma, pleural effusion
27	MDA-MB-361		Adenocarcinoma, breast, metastasis to brain

TABLE II continued... EMI50.1

ATTC NUMBER	HTB	CELL LINE	TUMOR TYPE
30	SK-BR-3		Adenocarcinoma, breast, malignant pleural effusion
31	C-33 A		Carcinoma, cervix

32 HT-3 Carcinoma, cervix, metastasis to lymph node
33 ME-180 Epidermoid carcinoma, cervix, metastasis to omentum
34 MS751 Epidermoid carcinoma, cervix, metastasis to lymph node
35 SiHa Squamous carcinoma, cervix
36 JEG-3 Choriocarcinoma
37 Caco-2 Adenocarcinoma, colon
38 HT-29 Adenocarcinoma, colon, moderately well-differentiated grade II
39 SK-CO-1 Adenocarcinoma, colon, ascites
40 HuTu 80 Adenocarcinoma, duodenum
41 A-253 Epidermoid carcinoma, submaxillary gland
43 FaDu Squamous cell carcinoma, pharynx
44 A-498 Carcinoma, kidney
45 A-704 Adenocarcinoma, kidney
46 Caki-1 Clear cell carcinoma, consistent with renal primary, metastasis to skin
47 Caki-2 Clear cell carcinoma, consistent with renal primary
48 SK-NEP-1 Wilms' tumor, pleural effusion
49 SW 839 Adenocarcinoma, kidney
52 SK-HEP-1 Adenocarcinoma, liver, ascites
53 A-427 Carcinoma, lung
54 Calu-l Epidermoid carcinoma grade III, lung, metastasis to pleura
55 Calu-3 Adenocarcinoma, lung, pleural effusion
56 Calu-6 Anaplastic carcinoma, probably lung

TABLE II continued... EMI51.1

ATTC HTB CELL LINE TUMOR TYPE NUMBER
57 SK-LU-1 Adenocarcinoma, lung consistent with poorly differentiated, grade III
58 SK-MES-1 Squamous carcinoma, lung, pleural effusion
59 SW 900 Squamous cell carcinoma, lung
60 EB1 Burkitt lymphoma, upper maxilla
61 EB2 Burkitt lymphoma, ovary
62 P3HR-1 Burkitt lymphoma, ascites
63 HT-144 Malignant melanoma, metastasis to subcutaneous tissue
64 Malme-3M Malignant melanoma, metastasis to lung
66 RPMI-7951 Malignant melanoma, metastasis to lymph node
67 SK-MEL-1 Malignant melanoma, metastasis to lymphatic system
68 SK-MEL-2 Malignant melanoma, metastasis to skin of thigh
69 SK-MEL-3 Malignant melanoma, metastasis to

lymph node
70 SK-MEL-5 Malignant melanoma, metastasis to axillary node
71 SK-MEL-24 Malignant melanoma, metastasis to node
72 SK-MEL-28 Malignant melanoma
73 SK-MEL-31 Malignant melanoma
75 Caov-3 Adenocarcinoma, ovary, consistent with primary
76 Caov-4 Adenocarcinoma, ovary, metastasis to subserosa of fallopian tube
77 SK-OV-3 Adenocarcinoma, ovary, malignant ascites
78 SW 626 Adenocarcinoma, ovary
79 Capan-I Adenocarcinoma, pancreas, metastasis to liver
80 ~ Capan-2 Adenocarcinoma, pancrease
TABLE II continued... EMI52.1

ATTC HTB CELL LINE TUMOR TYPE
NUMBER
81 DU 145 Carcinoma, prostate, metastasis to brain
82 A-204 Rhabdomyosarcoma
85 Saos-2 Osteogenic sarcoma, primary
86 SK-ES-1 Anaplastic osteosarcoma versus Ewing sarcoma, bone
88 SK-LMS-1 Leiomyosarcoma, vulva, primary
91 SW 684 Fibrosarcoma
92 SW 872 Liposarcoma
93 SW 982 Axilla synovial sarcoma
94 SW 1353 Chondrosarcoma, humerus
96 U-2 OS Osteogenic sarcoma, bone primary
102 Malme-3 Skin fibroblast
103 KATO III Gastric carcinoma
104 Cate-IB Embryonal carcinoma, testis, metastasis to lymph node
105 Tera-I Embryonal carcinoma, malignancy consistent with metastasis to lung
106 Tera-2 Embryonal carcinoma, malignancy consistent with, metastasis to lung
107 SW579 Thyroid carcinoma
111 AN3 CA Endometrial adenocarcinoma, metastatic
112 HEC-1-A Endometrial adenocarcinoma
113 HEC-1-B Endometrial adenocarcinoma
114 SK-UT-1 Uterine, mixed mesodermal tumor, consistent with leiomyosarcoma grade III
115 SK-UT-1B Uterine, mixed mesodermal tumor, consistent with leiomyosarcoma grade III
117 SW 954 Squamous cell carcinoma, vulva
118 SW 962 Carcinoma, vulva, lymph node metastasis

119 NCI-H69 Small cell carcinoma, lung
TABLE II continued... EMI53.1

ATTC HTB CELL LINE TUMOR TYPE NUMBER
120 NCI-H128 Small cell carcinoma, lung
121 BT-483 Ductal carcinoma, breast
122 BT-549 Ductal carcinoma, breast
123 DU4475 Metastatic cutaneous nodule, breast carcinoma
124 HBL-100 Breast
125 Hs 578Bst Breast, normal
126 Hs 578T Ductal carcinoma, breast
127 MDA-MB-330 Carcinoma, breast
128 MDA-MB-415 Adenocarcinoma, breast
129 MDA-MB-435S Ductal carcinoma, breast
130 MDA-MB-436 Adenocarcinoma, breast
131 MDA-MB-453 Carcinoma, breast
132 MDA-MB-468 Adenocarcinoma, breast
133 T-47D Ductal carcinoma, breast, pleural effusion
134 Hs 766T Carcinoma, pancreas, metastatic to lymph node
135 Hs 746T Carcinoma, stomach, metastatic to left leg
137 Hs 695T Amelanotic melanoma, metastatic to lymph node
138 Hs 683 Glioma
140 Hs 294T Melanoma, metastatic to lymph node
142 Hs 602 Lymphoma, cervical
144 JAR Choriocarcinoma, placenta
146 Hs 445 Lymphoid, Hodgkin's disease
147 Hs 700T Adenocarcinoma, metastatic to pelvis
148 H4 ~ Neuroglioma, brain
TABLE II continued... EMI54.1

ATTC HTB CELL LINE TUMOR TYPE NUMBER
151 Hs 696 Adenocarcinoma primary, unknown, metastatic to bone-sacrum
152 Hs 913T Fibrosarcoma, metastatic to lung
153 Hs 729 Rhabdomyosarcoma, left leg
157 FHs 738Lu Lung, normal fetus
158 FHs 173We Whole embryo, normal
160 FHs 738B1 Bladder, normal fetus
161 NIH::OVCAR Ovary, adenocarcinoma

-3
163 Hs 67 Thymus, normal
166 RD-ES Ewing's sarcoma
168 ChaGo K-I Bronchogenic carcinoma, subcutaneous metastasis, human
169 WERI-Rb-1 Retinoblastoma
171 NCI-H446 Small cell carcinoma, lung
172 NCI-H209 Small cell carcinoma, lung
173 NCI-H146 Small cell carcinoma, lung
174 NCI-H441 Papillary adenocarcinoma, lung
175 NCI-H82 Small cell carcinoma, lung
176 H9 T-cell lymphoma
177 NCI-H460 Large cell carcinoma, lung
178 NCI-H596 Adenosquamous carcinoma, lung
179 NCI-H676B Adenocarcinoma, lung
180 NCI-H345 Small cell carcinoma, lung
181 NCI-H820 Papillary adenocarcinoma, lung
182 NCI-H520 Squamous cell carcinoma, lung
183 NCI-H661 Large cell carcinoma, lung
184 NCI-H510A Small cell carcinoma, extra pulmonary origin, metastatic
185 D283 Med Medulloblastoma
186 Daoy Medulloblastoma
187 D341 Med Medulloblastoma
188 AML-193 Acute monocyte leukemia
189 ~ MV4-11 Leukemia biphenotype

(a) Anti-Tumor Cell Antibodies A straightforward means of recognizing a tumor antigen target is through the use of an antibody that has binding affinity for the particular antigen. An extensive number of antibodies are known that are directed against solid tumor antigens. Certain useful anti-tumor antibodies are listed above in Table I.

However, as will be instantly known to those of skill in the art, certain of the antibodies listed in Table I will not have the appropriate biochemical properties, or may not be of sufficient tumor specificity, to be of use therapeutically. An example is MUC8-22 that recognizes a cytoplasmic antigen. Antibodies such as these will generally be of use only in investigational embodiments, such as in model systems or screening assays.

Generally speaking, antibodies for use in these aspects of the present invention will preferably recognize antigens that are accessible on the cellsurface and that are preferentially, or specifically, expressed by tumor cells. Such antibodies will also preferably exhibit properties of high affinity, such as exhibiting a Kd of < 200 nM, and preferably, of < 100 nM, and will not show significant reactivity with lifesustaining normal tissues, such as one or more tissues selected from heart, kidney, brain, liver, bone marrow, colon, breast, prostate, thyroid, gall bladder, lung, adrenals, muscle, nerve fibers, pancreas, skin, or other life-sustaining organ or tissue in the human body.The "life-sustaining" tissues that are the most important for the purposes of the present invention, from the standpoint of low reactivity, include heart, kidney, central and peripheral nervous system tissues and liver.

The term "significant reactivity", as used herein, refers to an antibody or antibody fragment, that, when applied to the particular tissue under conditions suitable for immunohistochemistry, will elicit either no staining or negligible staining with only a few positive cells scattered among a field of mostly negative cells.

Particularly promising antibodies from Table I contemplated for use in the present invention are those having high selectivity for the solid tumor. For example, antibodies binding to TAG 72 and the HER-2 proto-oncogene protein, which are selectively found on the surfaces of many breast, lung and colorectal cancers (Thor et al., 1986; Colcher et al., 1987; Shepard et al., 1991); MOv18 and OV-TL3 and antibodies that bind to the milk mucin core protein and human milk fat globule (Miotti et awl., 1985; Burchell et awl., 1983); and the antibody 9.2.27 that binds to the high Mr melanoma antigens (Reisfeld et awl., 1982).Further useful antibodies are those against the folate-binding protein, which is known to be homogeneously expressed in almost all ovarian carcinomas; those against the erb family of oncogenes that are over-expressed in squamous cell carcinomas and the majority of gliomas; and other antibodies known to be the subject of ongoing pre-clinical and clinical evaluation.

The antibodies B3, KSI/4, CC49, 260F9, XMMCO-791, D612 and SM3 are believed to be particularly suitable for use in clinical embodiments, following the standard preclinical testing routinely practiced in the art. B3 (U.S. Patent 5,242,813; Brinkmann et al., 1991) has ATCC Accession No. HB 10573; KS1/4 can be made as described in U.S. Patent 4,975,369; and D612 (U.S. Patent 5,183,756) has ATCC Accession No. HB 9796.

Another means of defining a tumor-associated target is in terms of the characteristics of the tumor cell, rather than describing the biochemical properties of an antigen expressed by the cell. Accordingly, the inventors contemplate that any antibody that preferentially binds to a tumor cell listed in Table II may be used as the targeting component of a bispecific ligand. The preferential tumor cell binding is again based upon the antibody exhibiting high affinity for the tumor cell and not having significant reactivity with life-sustaining normal cells or tissues, as defined above.

The invention therefore provides several means for generating an antibody for use in the targeted coagulation methods described herein. To generate a tumor cell-specific antibody, one would immunize an animal with a composition comprising a tumor cell antigen and, as described more fully herein below, select a resultant antibody with appropriate specificity. The immunizing composition may contain a purified, or partially purified, preparation of any of the antigens in Table I; a composition, such as a membrane preparation, enriched for any of the antigens in Table I; any of the cells listed in Table II; or a mixture or population of cells that include any of the cell types listed in Table II.

Of course, regardless of the source of the antibody, in the practice of the invention in human treatment, one will prefer to ensure in advance that the clinicallytargeted tumor expresses the antigen ultimately selected.

This is achieved by means of a fairly straightforward assay, involving antigenically testing a tumor tissue sample, for example, a surgical biopsy, or perhaps testing for circulating shed antigen. This can readily be carried out in an immunological screening assay such as an ELISA (enzyme-linked immunosorbent assay), wherein the binding affinity of antibodies from a "bank" of hybridomas are tested for reactivity against the tumor.

Antibodies demonstrating appropriate tumor selectivity and affinity are then selected for the preparation of bispecific antibodies of the present invention.

Due to the well-known phenomenon of crossreactivity, it is contemplated that useful antibodies may result from immunization protocols in which the antigens originally employed were derived from an animal, such as a mouse or a primate, in addition to those in which the original antigens were obtained from a human cell. Where antigens of human origin are used, they may be obtained from a human tumor cell line, or may be prepared by obtaining a biological sample from a particular patient in question. Indeed, methods for the development of antibodies that are "custom-tailored" to the patient's tumor are known (Stevenson et al., 1990) and are contemplated for use in connection with this invention.

(b) Further Tumor Cell Targets and Binding Ligands In addition to the use of antibodies, other ligands could be employed to direct a coagulating agent to a tumor site by binding to a tumor cell antigen. For tumor antigens that are over-expressed receptors (oestrogen receptor, EGF receptor), or mutant receptors, the corresponding ligands could be used as targeting agents.

In an analogous manner to endothelial cell receptor ligands, there may be components that are specifically, or preferentially, bound to tumor cells. For example, if a tumor antigen is an over-expressed receptor, the tumor cell may be coated with a specific ligand in vivo. It seems that the ligand could then be targeted either with an antibody against the ligand, or with a form of the receptor itself. Specific examples of these type of targeting agents are antibodies against TIE-1 or TIE-2 ligands, antibodies against platelet factor 4, and leukocyte adhesion binding protein.

2. Other Disease Targets In further embodiments, the first binding region may be a component that binds to a target molecule that is specifically or preferentially expressed in a disease site other than a tumor site.

Exemplary target molecules associated with other diseased cells include, for example, leukocyte adhesion molecules, that are associated with psoriasis; FGF, that is associated with proliferative diabetic retinopathy; platelet factor 4, that is associated with the activated endothelium of various diseases; and VEGF, that is

associated with vascular proliferative disease. It is believed that an animal or patient having any one of the above diseases would benefit from the specific induction of coagulation in the disease site.

Diseases that are known to have a common angiodependent pathology, as described in Klagsburn & Folkman (1990), may also be treated with bispecific ligand as described herein. In particular, a vascular endothelial cell-targeted ligand or a stroma-targeted ligand will be used to achieve coagulation in the disease site. The treatment of BPH, diabetic retinopathy, vascular restenosis, vascular adhesions, AVM, meningioma, hemangioma, neovascular glaucoma, rheumatoid arthritis and psoriasis are particularly contemplated at the present time.

3. Disease-Associated Vasculature Cell Targets The cells of the vasculature are intended as targets for use in the present invention. In these cases, one binding region of the bispecific ligand will be capable of binding to an accessible marker preferentially expressed by disease-associated vasculature endothelial cells. The exploitation of the vascular markers is made possible due to the proximity of the vascular endothelial cells to the disease area and to the products of the local aberrant physiological processes. For example, tumor vascular endothelial cells are exposed to tumor cells and tumor-derived products that change the phenotypic profile of the endothelial cells.

Tumor cells are known to elaborate tumor-derived products, such as lymphokines, monokines, colonystimulating factors, growth factors and angiogenic factors, that act on the nearby vascular endothelial cells (Kandel et al., 1991; Folkman, 1985a,b) and cytokines (Burrows et awl., 1991; Ruco et a)., 1990; Borden eta)., 1990). The tumor products bind to the endothelial cells and serve to selectively induce expression of certain molecules. It is these induced molecules that may be targeted using the tumor endothelium-specific coagulant delivery provided by certain aspects of the present invention.Vascular endothelial cells in tumors proliferate at a rate 30-fold greater than those in miscellaneous normal tissues (Denekamp et awl., 1982), suggesting that proliferationlinked determinants could also serve as markers for tumor vascular endothelial cells.

In certain embodiments of the invention, the targeting component of the bispecific ligands will be a component that has a relatively high degree of specificity for tumor vasculature. These targeting components may be defined as components that bind to molecules expressed on tumor endothelium, but that have little or no expression at the surface of normal endothelial cells. Such specificity may be assessed by the standard procedures of immunostaining of tissue sections, which are routine to those of skill in the art.

However, as stated above, an advantage of the present invention is that the requirement for selectivity is not as stringent as previously needed in the prior art methods, especially those employing immunotoxins, because any side effects associated with the mis-targeting of the coagulating agent will be minimal in comparison to those resulting from the mis-targeting of a toxin.

Therefore, it is generally proposed that the molecules to be targeted using the bispecific ligands or antibodies of this invention will be those that are expressed on tumor vasculature at a higher level than on normal endothelial cells.

(a) Vascular Endothelial Cell Markers in Disease Molecules that are known to be preferentially expressed at the surface of vascular endothelial cells in a disease site or environment are herein termed natural disease-associated vascular endothelial cell markers".

This term is used for simplicity to refer to the endothelial cell components that are expressed in diseases connected with increased or inappropriate angiogenesis or endothelial cell proliferation. One particular example are the tumor endothelial cell components that are expressed in situ in response to tumor-derived factors. These components are also termed "naturally-induced tumor endothelial cell markers".

Both VEGF/VPF (vascular endothelial growth factor/vascular permeability factor) and components of the FGF (fibroblast growth factor) family are concentrated in or on tumor vasculature. The corresponding receptors therefore provide a potential target for attack on tumor vasculature. For example, VEGF receptors are known to be upregulated on tumor endothelial cells, as opposed to endothelial cells in normal tissues, both in rodents and man (Thieme et awl., 1995). Possibly, this is a consequence of hypoxia - a characteristic of the tumor microenvironment (Leith eta)., 1992). FGF receptors are also upregulated threefold on endothelial cells exposed to hypoxia, and so are believed to be upregulated in tumors (Bicknell

and Harris eta)., 1992).

The TGF p (transforming growth factor P) receptor (endoglin) on endothelial cells is upregulated on dividing cells, providing another target. One of the present inventors found that endoglin is upregulated on activated and dividing HUVEC in culture, and is strongly expressed in human tissues on endothelial cells at sites of neovascularization, including a broad range of solid tumors and fetal placenta. In contrast, endothelial cells in the majority of miscellaneous non-malignant adult tissues, including preneoplastic lesions, contain little or no endoglin.Importantly, endoglin expression is believed to correlate with neoplastic progression in the breast, as shown by benign fibroadenomas and early carcinomas binding low levels of TEC-4 and TEC-11 antibodies, and late stage intraductal carcinomas and invasive carcinomas binding high levels of these antibodies.

Other natural disease-associated vascular endothelial cell markers include a TIE, VCAM-1, Pselectin, E-selectin, avss3 integrin, pleiotropin and endosialin, each of which may be targeted using the invention.

(b) Cytokine-Inducible Vascular Endothelial Markers Due to the nature of disease processes, which often result in localized dysfunction within the body, methods are available to manipulate the disease site whilst leaving other tissues relatively unaffected. This is particularly true in malignant and benign tumors, which exist as distinct entities within the body of an animal.

For example, the tumor environment may be manipulated to create additional markers that are specific for tumor vascular endothelial cells. These methods generally mimic those that occur naturally in solid tumors, and also involve the local production of signalling agents, such as growth factors or cytokines, that induce the specific expression of certain molecules at the surface of the nearby vascular endothelial cells.

The group of molecules that may be artificially induced to be expressed at the surface of vascular endothelial cells in a disease or tumor environment are herein termed "inducible endothelial cell markers',, or specifically, inducible tumor endothelial cell markers.

This term is used to refer to those markers that are artificially induced, i.e., induced as a result of manipulation by the hand of man, rather than those that are induced as part of the disease or tumor development process in an animal. The term "inducible marker", as defined above, is chosen for simple referencetin the context of the present application, notwithstanding the fact that "natural markers" are also induced, e.g., by tumor-derived agents.

Thus, although not required to practice the invention, techniques for the selective elicitation of vascular endothelial antigen targets on the surface of disease-associated vasculature are available that may, if desired, be used in conjunction with the invention.

These techniques involve manipulating the antigenic expression, or cell surface presentation, such that a target antigen is expressed or rendered available on the surface of disease-associated vasculature and not expressed or otherwise rendered accessible or available for binding, or at least to a lesser extent, on the surface of normal endothelium.

Tumor endothelial markers can be induced by tumorderived cytokines (Burrows et awl., 1991; Ruco et awl., 1990) and by angiogenic factors (Mignatti et al., 1991).

Examples of cell surface markers that may be specifically induced in the tumor endothelium and then targeted using a bispecific coagulating ligand, as provided by the invention, include those listed in Table III (Bevilacqua et al., 1987; Dustin et al., 1986; Osborn et al., 1989; Collins et awl., 1984).

The mechanisms for the induction of the proposed markers; the inducing, or "intermediate cytokine", such as IL-1 and IFN-z; and the leukocyte cell type and associated cytokine-activating molecule, whose targeting will result in the release of the cytokine, are also set forth in Table III. In the induction of a specific marker, a bispecific "cytokine-inducing" or "antigeninducing" antibody is generally required. This antibody will selectively induce the release of the appropriate cytokine in the locale of the tumor, thus selectively inducing the expression of the desired target antigen by the vascular endothelial cells. The bispecific antibody cross-links cells of the tumor mass and cytokineproducing leukocytes, thereby activating the leukocytes to release the cytokine.

The preparation and use of bispecific antibodies such as these is predicated in part on the fact that cross-linking antibodies recognizing CD3, CD14, CD16 and CD28 have previously been shown to elicit cytokine production selectively upon cross-linking with the second antigen (Qian et al., 1991). In the context of the present invention, since only successfully tumor cellcrosslinked leukocytes will be activated to release the cytokine, cytokine release will be restricted to the locale of the tumor. Thus, expression of the desired marker, such as E-selectin, will be similarly limited to the endothelium of the tumor vasculature.

TABLE III POSSIBLE INDUCIBLE VASCULAR TARGETS EMI66.1

INDUCIBLE ACRONYN SUBTYPES/ALIASES INDUCING LEUKOCYTES LEUKOCYTE MOLECULES
ENDOTHELIAL (MOLECULAR FAMILY) CYTOKINES WHICH PRODUCE WHICH, WHEN CROSSLINKED
CELL THOSE CYTOKINES BY MONOCLONAL ANTIBODIES
MOLECULES ACTIVATE THE CELLS TO
PRODUCE CYTOKINES
Endothelial- ELAM-1 -- IL-1, TNF-α;, monocytas CD14
Leukocyte (Selectin) (TNF-ss)
macrophages CD14
Adhesion E-select-in (Bacterial
Molecule-1 Endotoxin) mast cells FcR for IgE
Vascular Cell VCAM-1 Inducible Cell Adhesion (Bacterial monocytes CD14
Adhesion Molecule-110 (INCAM- Endotoxin) IL-1,
macrophages CD14
Molecule-1 110) TNF-α;
(Immunoglobulin Family) mast cells FcR for IgE
TNF-ss, IL-4 helper T cells CD2, CD3, CD28
TNF NK cells FcR for IgG (CD16)
TABLE III continued... EMI67.1

INDUCIBLE ACRONYN SUBTYPES/ALIASES INDUCING LEUKOCYTES LEUKOCYTE MOLECULES
ENDOTHELIAL (MOLECULAR FAMILY) CYTOKINES WHICH PRODUCE WHICH, WHEN CROSSLINKED
CELL THOSE CYTOKINES BY MONOCLONAL ANTIBODIES
MOLECULES ACTIVATE THE CELLS TO
PRODUCE CYTOKINES
Intercellular ICAM-1 -- IL-1, TNFα monocytes CD14
Adhesion (Immunoglobulin Family) (Bacterial
macrophages CD15
Molecule-1 Endotoxin)
mast cells FcR for IgE
TNF-ss, IFNγ T helper cells CD2, CD3, CD28
NK cells FcR for IgG (CD16)
The Agent for LAM-1 MEL-14 Agent (Mouse) II-1, TNFα monocytes CD14
Leukocyte Agent (Bacterial
macrophages CD14
Adhesion Endotoxin)
Molecule-1 mast cells FcR for IgE
Major MHC Class HLA-DR IFN-γ helper T cells CD2, CD3, CD28
Histocompatabil II HLA-DP - Human
ity Complex HLA-DQ
Class II
NK cells FcR for IgG (CD16)
Antigen I-A - Mouse
I-E

It is important to note that, from the possible inducible markers listed in Table III, E-selectin and MMC Class II antigens, such as HLA-DR, HLA-DP and HLA-DQ (Collins et awl., 1984), are by far the most preferred

targets for use in connection with clinical embodiments.

The other adhesion molecules of Table III appear to be expressed to varying degrees in normal tissues, generally in lymphoid organs and on endothelium, making their targeting perhaps appropriate only in animal models or in cases where their expression on normal tissues can be inhibited without significant side-effects. The targeting of E-selectin or an MMC Class II antigen is preferred as the expression of these antigens will likely be the most direct to promote selectively in tumorassociated endothelium.

B-seiectin The targeting of an antigen that is not expressed on the surfaces of normal endothelium is the most straightforward form of the induction methods.

E-selectin is an adhesion molecule that is not expressed in normal endothelial vasculature or other human cell types (Cotran et al., 1986), but can be induced on the surface of endothelial cells through the action of cytokines such as IL-1, TNF, lymphotoxin and bacterial endotoxin (Bevilacqua et al., 1987). It is not induced by IFN- (Wu et al., 1990). The expression of E-selectin may thus be selectively induced in tumor endothelium through the selective delivery of such a cytokine, or via the use of a composition that causes the selective release of such cytokines in the tumor environment.

Bispecific antibodies are one example of a composition capable of causing the selective release of one or more of the foregoing or other appropriate cytokines in the tumor site, but not elsewhere in the body. Such bispecific antibodies are herein termed "antigen-inducing antibodies" and are, of course, distinct from any bispecific antibodies of the invention that have targeting and coagulating components. Antigeninducing antibodies are designed to cross-link cytokine effector cells, such as cells of monocyte/macrophage lineage, T cells and/or NK cells or mast cells, with tumor cells of the targeted solid tumor mass. This cross-linking would then effect a release of cytokine that is localized to the site of cross-linking, i.e., the tumor.

Effective antigen-inducing antibodies recognize a selected tumor cell surface antigen on the one hand (e.g., those in Table I) and, on the other hand, recognize a selected "cytokine activating" antigen on the surface of a selected leukocyte cell type. The term "cytokine activating" antigen is used to refer to any one of the various known molecules on the surfaces of leukocytes that, when bound by an effector molecule, such as an antibody or a fragment thereof or a naturallyoccurring agent or synthetic analog thereof, be it a soluble factor or membrane-bound counter-receptor on another cell, promotes the release of a cytokine by the leukocyte cell.Examples of cytokine activating molecules include CD14 (the LPS receptor) and FcR for IgE, which will activate the release of IL-1 and TNFa; and CD16, CD2 or CD3 or CD28, which will activate the release of IFNy and TNFss, respectively.

Once introduced into the bloodstream of an animal bearing a tumor, such an antigen-inducing bispecific antibody will bind to tumor cells within the tumor, cross-link those tumor cells with effector cells, e.g., monocytes/macrophages, that have infiltrated the tumor, and thereafter effect the selective release of cytokine within the tumor. Importantly, however, without crosslinking of the tumor and leukocyte, the antigen-inducing antibody will not effect the release of cytokine. Thus, no cytokine release will occur in parts of the body removed from the tumor and, hence, expression of cytokine-induced molecules, e.g., E-selectin, will occur only within the tumor endothelium.

A number of useful "cytokine activating" antigens are known, which, when cross-linked with an appropriate bispecific antibody, will result in the release of cytokines by the cross-linked leukocyte. The generally preferred target for this purpose is CD14, which is found on the surface of monocytes and macrophages. When CD14 is cross linked it stimulates monocytes/ macrophages to release IL-1 (Schutt et awl., 1988; Chen et awl., 1990), and possibly other cytokines, which, in turn stimulate the appearance of E-selectin on nearby vasculature.

Other possible targets for cross-linking in connection with E-selectin induction and targeting include FcR for IgE, found on Mast cells; FcR for IgG (CD16), found on NK cells; as well as CD2, CD3 or CD28, found on the surfaces of T cells. Of these, CD14 targeting is generally preferred due to the relative prevalence of monocyte/ macrophage infiltration of solid tumors as opposed to the other leukocyte cell types.

In an exemplary induction embodiment, an animal bearing a solid tumor is injected with bispecific (Fab' Fab') anti-CD14/anti-tumor antibody (such as anti-CEA, 9.2.27 antibody against high Mr melanoma antigens OV-TL3 or MOv 18 antibodies against ovarian associated antigens). The antibody localizes in the tumor, by virtue of its tumor binding activity, and then activates monocytes and macrophages in the tumor by

crosslinking their CD14 antigens (Schutt et. al., 1988; Chen et. al., 1990). The activated monocytes/macrophages have tumoricidal activity (Palleroni et. al., 1991) and release IL-1 and TNF which rapidly induce E-selectin antigens on the tumor vascular endothelial cells (Bevilacqua et. al., 1987; Pober et. al., 1991).

MHC Class II Antigens The second preferred group of inducible markers contemplated for use with the present invention are the MMC Class II antigens (Collins et al., 1984), including HLA-DR, HLA-DP and HLA-DQ. Class II antigens are expressed on vascular endothelial cells in most normal tissues in several species, including man. Studies in vitro (Collins et al., 1984; Daar et al., 1984; O'Connell et al., 1990) and in vivo (Groenewegen et al., 1985) have shown that the expression of Class II antigens by vascular endothelial cells requires the continuous presence of IFN-r which is elaborated by TH1 cells and, to a lesser extent, by NK cells and CD8+ T cells.

MMC Class II antigens are not unique to vascular endothelial cells, and are also expressed constitutively on B cells, activated T cells, cells of monocyte/macrophage linage and on certain epithelial cells, both in mice (Hammerling, 1976) and in man (Daar et al., 1984). Due to the expression of MMC Class II antigens on "normal" endothelium, their targeting is not quite so straightforward as E-selectin. However, the induction and targeting of MMC Class II antigens is made possible by using in conjunction with an immunosuppressant, such as Cyclosporin A (CsA), that has the ability to effectively inhibit the expression of Class II molecules in normal tissues (Groenewegen et al., 1985).The CsA acts by preventing the activation of T cells and NK cells (Groenewegen et awl., 1985; DeFranco, 1991), thereby reducing the basal levels of IFN-y below those needed to maintain Class II expression on endothelium.

There are various other cyclosporins related to CsA, including cyclosporins A, B, C, D, G, and the like, that also have immunosuppressive action and are likely to demonstrate an ability to suppress Class II expression.

Other agents that might be similarly useful include FK506 and rapamycin.

Thus, the practice of the MMC Class II induction and targeting embodiment requires a pretreatment of the tumor-bearing animal with a dose of CsA or other Class II immunosuppressive agent that is effective to suppress Class II expression. In the case of CsA, this will typically be on the order of about 10 to about 30 mg/kg body weight. Once suppressed in normal tissues, Class II antigens can then be selectively induced in the tumor endothelium, again through the use of a bispecific antibody.

In this case, the antigen-inducing bispecific antibody will have specificity for a tumor cell marker and for an activating antigen found on the surface of an effector cell that is capable of inducing IFNproduction. Such effector cells will generally be helper T cells (TH) or Natural Killer (NK) cells. In these embodiments, it is necessary that T cells, or NK cells if CD16 is used, be present in the tumor to produce the cytokine intermediate in that Class II antigen expression is achieved using IFN-, but is not achieved with the other cytokines. Thus, for the practice of this aspect of the invention, one will desire to select CD2, CD3, CD28, or most preferably CD28, as the cytokine activating antigen for targeting by the antigen-inducing bispecific antibody.

The T cells that should be activated in the tumor are those adjacent to the vasculature since this is the region most accessible to cells and is also where the bispecific antibody will be most concentrated. The activated T cells should then secrete IFN-y which induces Class II antigens on the adjacent tumor vasculature.

The use of a bispecific (Fab'-Fab') antibody having one arm directed against a tumor antigen and the other arm directed against CD28 is currently preferred. This antibody will crosslink CD28 antigens on T cells in the tumor which, when combined with a second signal (provided, for example, by IL-1 which is commonly secreted by tumor cells (Burrows et awl., 1991; Ruco et al., 1990), has been shown to activate T cells through a CA2+-independent non-CsA-inhibitable pathway (Hess et al., 1991; June et al., 1987; Bjorndahl et al., 1989).

The preparation of antibodies against various cytokine activating molecules is also well kndwn in the art. For example, the preparation and use of anti-CD14 and anti-CD28 monoclonal antibodies having the ability to induce cytokine production by leukocytes has now been described by several laboratories (reviewed in Schutt et awl., 1988; Chen et awl., 1990, and June et awl., 1990, respectively). Moreover, the preparation of

monoclonal antibodies that will stimulate leukocyte release of cytokines through other mechanisms and other activating antigens is also known (Clark et awl., 1986; Geppert et al., 1990).

In still further embodiments, the inventors contemplate an alternative approach for suppressing the expression of Class II molecules, and selectively eliciting Class II molecule expression in the locale of the tumor. This approach, which avoids the use of both CsA and a bispecific activating antibody, takes advantage of the fact that the expression of Class II molecules can be effectively inhibited by suppressing IFN- production by T cells, e.g., through use of an anti-CD4 antibody (Street et al., 1989). Using this embodiment, IFNproduction is inhibited by administering anti-CD4, resulting in the general suppression of Class II expression. Class II is then induced only in the tumor site, e.g., using tumor-specific T cells which are only activatable within the tumor.

In this mode of treatment, one will generally pretreat an animal or human patient with a dose of anti CD4 that is effective to suppress IFN-y production and thereby suppress the expression of Class II molecules.

Effective doses are contemplated to be, for example, on the order of about 4 to about 10 mg/kg body weight.

After Class II expression is suppressed, one will then prepare and introduce into the bloodstream an IFN-T-producing T cell clone (e.g., Thl or cytotoxic T lymphocyte, CTL) specific for an antigen expressed on the surface of the tumor cells. These T cells localizes to the tumor mass, due to their antigen recognition capability and, upon such recognition, then release IFN W. In this manner, cytokine release is again restricted to the tumor, thus limiting the expression of Class II molecules to the tumor vasculature.

The IFN-y-producing T cell clone may be obtained from the peripheral blood (Mazzocchi et al., 1990), however, a preferred source is from within the tumor mass (Fox et al., 1990). The currently preferred means of preparing such a T cell clone is to remove a portion of the tumor mass from a patient; isolate cells, using collagenase digestion, where necessary; enrich for tumor infiltrating leukocytes using density gradient centrifugation, followed by depletion of other leukocyte subsets by, e.g., treatment with specific antibodies and complement; and then expand the tumor infiltrating leukocytes in vitro to provide the IFN- producing clone.

This clone will necessarily be immunologically compatible with the patient, and therefore should be well tolerated by the patient.

It is proposed that particular benefits will be achieved by further selecting a high IFN-y producing T cell clone from the expanded leukocytes by determining the cytokine secretion pattern of each individual clone every 14 days. To this end, rested clones will be mitogenically or antigenically-stimulated for about 24 hours and their culture supernatants assayed, e.g., using a specific sandwich ELISA technique (Cherwinski et awl., 1989), for the presence of IL-2, IFN- , IL-4, IL-5 and IL-10. Those clones secreting high levels of IL-2 and IFN-y, the characteristic cytokine secretion pattern of TH1 clones, will be selected. Tumor specificity will be confirmed using proliferation assays.

Furthermore, one will prefer to employ as the anti-CD4 antibody an anti-CD4 Fab, because it will be eliminated from the body within 24 hours after injection and so will not cause suppress ion of the tumorrecognizing T-cell clones that are subsequently administered. The preparation of T cell clones having tumor specificity is generally known in the art, as exemplified by the production and characterization of T cell clones from lymphocytes infiltrating solid melanoma tumors (Maeda eta)., 1991).

In using either of the MMC Class II suppressioninduction methods, additional benefits will likely result from the fact that anti-Class II antibodies injected intravenously do not appear to reach the epithelial cells or the monocytes/macrophages in normal organs other than the liver and spleen. Presumably this is because the vascular endothelium in most normal organs is tight, not fenestrated as it is in the liver and spleen, and so the antibodies must diffuse across basement membranes to reach the Class II-positive cells. Also, any B cell elimination that may result, e.g., following crosslinking, is unlikely to pose a significant problem as these cells are replenished from Class II negative progenitors (Lowe et awl., 1986). Even B cell killing, as occurs in B lymphoma patients, causes no obvious harm (Vitetta et al., 1991).

In summary, although the tumor coagulating compositions and antibodies of the present invention are elegantly simple, and do not require the induction of antigens for their operability, the combined use of an

antigen-inducing bispecific antibody with this invention is also contemplated. Such antibodies would generally be administered prior to the bispecific coagulating ligands of this invention.

Generally speaking, the more "immunogenic" tumors would be more suitable for the MMC Class II approach involving, e.g., the cross-linking of T cells in the tumor through an anti-CD28/anti-tumor bispecific antibody, because these tumors are more likely to be infiltrated by T cells, a prerequisite for this method to be effective. Examples of immunogenic solid tumors include renal carcinomas, melanomas, a minority of breast and colon cancers, as well as~possibly pancreatic, gastric, liver, lung and glial tumor cancers. These tumors are referred to as "immunogenic" because there is evidence that they elicit immune responses in the host and they have been found to be amenable to cellular immunotherapy (Yamaue et al., 1990).In the case of melanomas and large bowel cancers, the most preferred antibodies for use in these instances would be B72.3 (anti-TAG-72) and PRSC5/PR4C2 (anti-Lewis a) or 9.2.27 (anti-high Mr melanoma antigen).

For the majority of solid tumors of all origins, an anti-CD14 approach that employs a macrophage/monocyte intermediate would be more suitable. This is because most tumors are rich in macrophages. Examples of macrophage-rich tumors include most breast, colon and lung carcinomas. Examples of preferred anti-tumor antibodies for use in these instances would be anti HER 2, B72.3, SM-3, HMFG-2, and SWA11 (Smith et al., 1989).

(c) Coagulant-Inducible Markers Coagulants, such as thrombin, Factor IX/IXa, Factor X/Xa, plasmin and metalloproteinases, such as interstitial collagenases, stromelysins and gelatinases, also act to induce certain markers. In particular, E-selectin, P-selectin, PDGF and ICAM-1 are induced by thrombin (Sugama et. awl., 1992; Shankar et. awl., 1994).

Therefore, for this induction, an anticoagulant/anti-tumor bispecific antibody will be utilized. The antibody will localize in the tumor via its tumor binding activity. The bispecific will then concentrate the coagulant, e.g., thrombin, in the tumor, resulting in induction of E-selectin and P-selectin on the tumor vascular endothelial cells (Sugama et. al., 1991; Shankar et. awl., 1994).

Alternatively, targeting of truncated tissue factor to tumor cells or endothelium will induce thrombin deposition within the tumor. As the thrombin is deposited, E-selectin and P-selectin will be induced on the tumor vascular endothelial cells.

(d) Antibodies to Vascular Endothelial Cell Markers A straightforward means of recognizing a diseaseassociated vasculature target, whether induced in the natural environment or by artificial means, is through the use of an antibody that has binding affinity for the particular cell surface receptor, molecule or antigen.

These include antibodies directed against all cell surface components that are known to be present on, e.g., tumor vascular endothelial cells, those that are induced or over-expressed in response to tumor-derived factors, and those that are induced following manipulation by the hand of man. Table IV and Table V summarize useful antibodies and their properties.

TABLE IV SUMMARY OF VASCULATURE STAINING PATTERNS OF CERTAIN ANTIBODIES TO HUMAN TUMOR VASCULATURE EMI79.1

Antibody	Antigen	Reference	% Tumor types stained	% tumor vessels stained	normal vessel reactivity
anti-vWF	VIII R Ag	100	100	strong	on all
FB5	endosialin	Rettig & old	30	10-20	lymphoid organs
TP3	80 kDa osteosarcoma related antigen	Bruland	50	10-30	strong on small BV protein
BC-1	fibronectin isoform	Zardi	60	10-30	none
TV-1	fibronectin	Epstein	100	100	strong on all
LM 609	*α*;vsse receptor	vitronectin	Cheneoh	85	70-80 medium on all
TEC 11	endoglin	Thorpe;	100	100	weak on most

TEC 110 VEGF Thorpe; 100 100 weak on most
TABLE V COMPARISON OF ANTI-EC mAbs ON HUMAN TUMORS EMI80.1

TUMOR TYPE n TEC 110 TEC 11 FB-5 TP-3 BC-1 TV-1 LM-609
DIGESTIVE
Gastrointestinal 9 ++ ++ +-- ++ + ++ ++
Parotid 3 ++ ++ - ++ (SMALL) - ND ND
REPRODUCTIVE
Breast 1 + ++ - ND ++ ++ Ovary 4 ++ ++ - ++ (SMALL) ++ ++ +
Uterus 2 ++ ++ - ++ ++ +
RESPIRATORY
Lung 3 ++ ++ + ND ++ ++ +
LYMPHOID
Hodgkins 2 ++ ++ - + - +-++ +
Two further antibodies that may be used in this invention are those described by Rettig et al. (1992) and Wang et a). (1993) that are directed against unrelated antigens of unknown function expressed in the vasculature of human tumors, but not in most normal tissues.

The antibody described by Kim et. a). (1993) may also be used in this invention, particularly as this antibody inhibited angiogenesis and suppressed tumor growth in vivo.

Antibodies that have not previously been shown to be specific for human tumors may also be used. For example, Venkateswaran et al. (1992) described the production of anti-FGF MAbs. Xu et. al. (1992) developed and characterized a panel of 16 isoform and domain-specific polyclonal and monoclonal antibodies against FGF receptor (flg) isoforms. Massoglia et al. (1987) also reported MAbs against FGF.

(e) Generation of Antibodies to Disease Vasculature In addition to utilizing a known antibody, such as those described above and others known and published in the scientific literature, one may also generate a novel antibody using standard immunization procedures, as described in more detail hereinbelow. To generate an antibody against a known disease-associated vascular marker antigen, one would immunize an animal with an immunogenic composition comprising the antigen. This may be a membrane preparation that includes, or is enriched for, the antigen; a relatively purified form of the antigen, as isolated from cells or membranes; a highly purified form of the antigen, as obtained by a variety of purification steps using, e.g., a native antigen extract or a recombinant form of the antigen obtained from a recombinant host cell.

The present invention also provides yet further methods for generating an antibody against an antigen present on disease-associated vasculature endothelial cells, which methods are suitable for use even where the biochemical identity of the antigen remains unknown.

These methods are exemplified through the generation of an antibody against tumor vasculature endothelial cells.

A first means of achieving antibody generation in this manner uses a preparation of vascular endothelial cells obtained from the tumor site of an animal or human patient. One simply immunizes an experimental animal with a preparation of such cells and collects the antibodies so produced. The most useful form of this method is that where specific antibodies are subsequently selected, as may be achieved using conventional hybridoma technology and screening against tumor vascular endothelial cells.

A development of the above method is that which mimics the tumor vasculature phenomenon in vitro, and where cell purification is not necessary. In using this method, endothelial cells are subjected to tumor-derived products, such as might be obtained from tumorconditioned media, in cell culture rather than in an animal. This method generally involves stimulating endothelial cells with tumor-conditioned medium and employing the stimulated endothelial cells as immunogens to prepare a collection of antibodies. Again, specific antibodies should be selected, e.g., using conventional monoclonal antibody technology, or other techniques such as combinatorial immunoglobulin phagemid libraries prepared from RNA isolated from the spleen of the immunized animal.One would select a specific antibody that preferentially recognizes tumor-stimulated vascular endothelium and reacts more strongly with tumorassociated endothelial cells than with normal adult human tissues.

Stimulated endothelial cells contemplated to be of use in this regard include, for example, human umbilical

vein endothelial cells (HUVE), human dermal microvascular endothelial cells (HDEMC), human saphenous vein endothelial cells, human omental fat endothelial cells, other human microvascular endothelial cells, human brain capillary endothelial cells, and the like. It is also contemplated that endothelial cells from another species may stimulated by tumor-conditioned media and employed as immunogens to generate hybridomas to produce an antibodies in accordance herewith, i.e., to produce antibodies that crossreact with tumor-stimulated human vascular endothelial cells, and/or antibodies for use in pre-clinical models.

"Tumor-conditioned medium or media" are defined herein as compositions or media, such as culture media, that contain one or more tumor-derived cytokies, lymphokines or other effector molecules. Most typically, tumor-conditioned medium is prepared from a culture medium in which selected tumor cells have been grown, and will therefore be enriched in such tumor-derived products. The type of medium is not believed to be particularly important, so long as it at least initially contains appropriate nutrients and conditions to support tumor cell growth. It is also, of course, possible to extract and even separate materials from tumorconditioned media and employ one or more of the extracted products for application to the endothelial cells.

As for the type of tumor used for the preparation of the medium or media, one will, of course, prefer to employ tumors that mimic or resemble the tumor that will ultimately be subject to analysis or treatment using the present invention. Thus, for example, where one envisions the development of a protocol for the treatment of breast cancer, one will desire to employ breast cancer cells such as ZR-75-1, T47D, SKBR3, MDA-MB-231. In the case of colorectal tumors, one may mention by way of example the HT29 carcinoma, as well as DLD-1, HCTll6 or even SW48 or SW122. In the case of lung tumors, one may mention by way of example NCI-H69, SW2, NCI H23, NCI H460, NCI H69, or NCI H82.In the case of melanoma, good examples are DX.3, A375, SKMEL-23, HMB-2, MJM, T8 or indeed VUP. In any of the above cases, it is further believed that one may even employ cells produced from the tumor that is to be treated, i.e., cells obtained from a biopsy.

Once prepared, the tumor-conditioned media is then employed to stimulate the appearance of tumor endothelium-specific marker(s) on the cell surfaces of endothelial cells, e.g., by culturing selected endothelial cells in the presence of the tumorconditioned media (or products derived therefrom).

Again, it is proposed that the type of endothelial cell that is employed is not of critical importance, so long as it is generally representative of the endothelium associated with the vasculature of the particular tumor that is ultimately to be treated or diagnosed. The inventors prefer to employ human umbilical vein endothelial cells (HUVE), or human dermal microvascular endothelial cells (HDMEC, Karasek, 1989), in that these cells are of human origin, respond to cytokine growth factors and angiogenic factors and are readily obtainable. However, it is proposed that any endothelial cell that is capable of being cultured in vitro may be employed in the practice of the invention and nevertheless achieve beneficial results. One may mention, by way of example, cells such as EA.hy9.26, ECV304, human saphenous vein endothelial cells, and the like.

Once stimulated using the tumor-derived products, the endothelial cells are then employed as immunogens in the preparation of monoclonal antibodies (MAbs). The technique for preparing MAbs against antigenic cell surface markers is quite straightforward, and may be readily carried out using techniques well known to those of skill in the art, as exemplified by the technique of Kohler & Milstein (1975), and further described hereinbelow.

Generally speaking, a preferred method of preparing MAbs using stimulated endothelial cells involves the following procedures: Cells or cell lines derived from human tumors are grown in tissue culture for 2 4 days.

The tissue culture supernatant ('tumor-conditioned medium') is removed from the tumor cell cultures and added to cultures of HUVEC at a final concentration of 50% (v/v). After 2 days culture the HUVEC are harvested non-enzymatically and 1-2 X 106 cells injected intraperitoneally into mice. This process is repeated three times at two-weekly intervals, the final immunization being by the intravenous route. Three days later the spleen cells are harvested and fused with SP2/0 myeloma cells by standard protocols (Kohler & Milstein, 1975) and hybridomas producing antibodies with the appropriate reactivity are cloned by limiting dilution.

From the resultant collection of hybridomas, one will then desire to select one of more hybridomas that produce an antibody that recognizes the activated vascular endothelium to a greater extent than it recognizes non-activated vascular endothelium. One goal is the identification of antibodies having virtually

no binding affinity for normal endothelium. However, in contrast to the prior art, in the present invention this property is not critical. In any event, one will generally identify suitable antibody-producing hybridomas by screening using, e.g., an ELISA, RIA, IRMA, IIF, or similar immunoassay, against one or more types of tumoractivated endothelial cells. Once candidates have been identified, one will desire to test for the absence of reactivity for non-activated or "normal" endothelium or other normal tissue or cell type.In this manner, hybridomas producing antibodies having an undesirably high level of normal cross-reactivity for the particular application envisioned may be excluded.

(f) Anti-Endoglin Antibodies Using the technique described above, antibodies having relative specificity for tumor vascular endothelium have been prepared and isolated. In one particular example, HT29 carcinoma cells were employed to prepare the conditioned medium, which was then employed to stimulate HUVE cells in culture. The resultant HT29activated HUVE cells were then employed as immunogens in the preparation of a hybridoma bank, which was ELISAscreened using HT29-activated HUVE cells and by immunohistologic analysis of sections of human tumors and normal tissues. From this bank, antibodies that recognized a tumor vascular endothelial cell antigen were selected.

The MAbs termed tumor endothelial cell antibody 4 and tumor endothelial cell antibody 11 (TEC4 and TEC11) were obtained using the above method. The antigen recognized by TEC4 and TEC11 was ultimately determined to be the molecule endoglin. The epitopes on endoglin recognized by TEC4 and TEC11 are present on the cell surface of stimulated HWE cells, and only minimally present (or immunologically accessible) on the surface of non-stimulated cells. MAbs have previously been raised against endoglin. However, analyzing the reactivity with HWEC or TCM-activated HWEC cell surface determinants by FACS or indirect immunofluorescence shows the epitopes recognized by TEC-4 and TEC-11 to be distinct from those of a previous antibody termed 44G4 (Gougos & Letarte, 1988).

Although any of the known anti-endoglin antibodies (e.g., Gougos & Letarte, 1988; Gougos et al., 1992; O'Connell et awl., 1992; Burring et awl., 1991) may be used in connection with the present invention, the TEC-4 and TEC-11 mAbs are envisioned to be particularly suitable.

This is because they label capillary and venular endothelial cells moderately to strongly in a broad range of solid tumors (and in several chronic inflammatory conditions and fetal placenta), but display relatively weak staining of vessels in the majority of normal, healthy adult tissues. TEC-11 is particularly preferred as it shows virtually no reactivity with non-endothelial cells. Furthermore, both TEC-4 and TEC-11 are complement-fixing, which imparts to them the potential to also induce selective lysis of endothelial cells in the tumor vascular bed.

Antibodies that are cross-reactive with the MAbs TEC-4 and TEC-11, i.e., those that bind to endoglin at the same epitope as TEC-4 or TEC-11, are also contemplated to be of use in this invention. The identification of an antibody or antibodies that bind to endoglin at the same epitopes as TEC-4 or TEC-11 is a fairly straightforward matter. This can be readily determined using any one of variety of immunological screening assays in which antibody competition can be assessed. For example, where the test antibodies to be examined are obtained from a different source to that of TEC-4 or TEC-11 , e.g., a rabbit, or are even of a different isotype, for example, IgG1 or IgG3, a competition ELISA may be employed.In one such embodiment of a competition ELISA one would pre-mix TEC-4 or TEC-11 with varying amounts of the test antibodies prior to applying to the antigen-coated wells in the ELISA plate. By using either anti-murine or anti-IgM secondary antibodies one will be able to detect only the bound TEC-4 or TEC-11 antibodies - the binding of which will be reduced by the presence of a test antibody that recognizes the same epitope as either TEC-4 or TEC-11.

To conduct an antibody competition study between TEC-4 or TEC-11 and any test antibody, one may first label TEC-4 or TEC-11 with a detectable label, such as, e.g., biotin or an enzymatic or radioactive label, to enable subsequent identification. In these cases, one would incubate the labelled antibodies with the test antibodies to be examined at various ratios (e.g., 1:1, 1:10 and 1:100) and, after a suitable period of time, one would then assay the reactivity of the labelled TEC-4 or TEC-11 antibodies and compare this with a control value in which no potentially competing antibody (test) was included in the incubation.

The assay may be any one of a range of immunological assays based upon antibody binding and the TEC-4 or TEC11 antibodies would be detected by means of detecting their label, e.g., using streptavidin in the case of biotinylated antibodies or by using a chromogenic substrate in connection with an enzymatic label or by simply detecting the radiolabel. An antibody that binds to the same epitope as TEC-4 or TEC-11 will be

able to effectively compete for binding and thus will significantly reduce TEC-4 or TEC-11 binding, as evidenced by a reduction in labelled antibody binding.

In the present case, after mixing the labelled TEC-4 or TEC-11 antibodies with the test antibodies, suitable assays to determine the remaining reactivity include, e.g., ELISAs, RIAs or western blots using human endoglin; immunoprecipitation of endoglin; ELISAs, RIAs or immunofluorescent staining of recombinant cells expressing human endoglin; indirect immunofluorescent staining of tumor vasculature endothelial cells; reactivity with HUVEC or TCM-activated HWEC cell surface determinants indirect immunofluorescence and FACS analysis. This latter method is most preferred and was employed to show that the epitopes recognized by TEC-4 and TEC-11 are distinct from that of 44G4 (Gougos & BR< Letarte, 1988).

The reactivity of the labelled TEC-4 or TEC-11 antibodies in the absence of any test antibody is the control high value. The control low value is obtained by incubating the labelled antibodies with unlabelled antibodies of the same type, when competition would occur and reduce binding of the labelled antibodies. A significant. reduction in labelled antibody reactivity in the presence of a test antibody is indicative of a test antibody that recognizes the same epitope, i.e., one that Across-reacts" with the labelled antibody.A "significant reduction" in this aspect of the present application may be defined as a reproducible (i.e., consistently observed) reduction in binding of at least about 10es-50k at a ratio of about 1:1, or more preferably, of equal to or greater than about 90% at a ratio of about 1:100.

The use of "cross-reactivity assays", as described above in the context of TEC-4 and TEC-11 antibodies, may be applied to any antibody for use in the present invention. Therefore, antibodies that bind to a component of a tumor cell, a component of tumor vasculature, a tumor cell-associated component, a tumor vasculature-associated component, a tumor extracellular matrix component, or to any cell type listed herein, at the same epitope as any of the antibodies listed herein, as determined by an antibody competition assay, will be an antibody that falls under the scope of this invention when combined with a coagulating agent to form a bispecific ligand.

(g) Use of Vascular Endothelial Cell Binding Ligands Biological ligands that are known to bind or interact with endothelial cell surface molecules, such as growth factor receptors, may also be employed as a targeting component.

The growth factors or ligands contemplated to be useful as targets in this sense include VEGF/VPF, FGF, TGFP, ligands that bind to a TIE, tumor-associated fibronectin isoforms, scatter factor, hepatocyte growth factor (HGF), platelet factor 4 (PF4), PDGF and TIMP.

Particularly preferred targets are VEGF/VPF, the FGF family of proteins and TGFss. Abraham et al. (1986) cloned FGF, which is therefore available as a recombinant protein. As reported by Ferrara et al. (1991), four species of VEGF having 121, 165, 189, and 206 amino acids have been cloned.

(h) Targeting of Bound Ligands Antibodies or specific targeting ligands may also be directed to any component that binds to the surface of vascular endothelial cells in a disease site, such as a tumor. Such components are exemplified by tumor-derived ligands and antigens, such as growth factors, that bind to specific cell surface receptors already present on the endothelial cells, or to receptors that have been induced, or over-expressed, on such cells in response to the tumor environment. Tumor vasculature-associated targets may also be termed tumor-derived endothelial cell binding factors.

Level of.specificity required for successful disease targeting will be achieved partly because the local endothelial cells will be induced to express, or reveal, receptors that are not present, or are underexpressed or masked, on normal endothelial cells. With tumors, further specificity will result due to the fact that endothelial cells in the tumor will capture the tumor-derived factors, and bind them to the cell surface, reducing the amount of ligand available for other tissues. When combined with the further dilution of the factor or ligand by distribution in the blood and tissue fluid pool, endothelial cells in normal tissues will be expected to bind relatively little of such factors.

Thus, operationally, cell-surface bound ligands or factors will be able to used as a tumor endothelial cell marker.

In addition to manufacture by the tumor cells themselves, tumor endothelial cell binding factors may also originate from other cell types, such as macrophages and mast cells, that have infiltrated tumors, or may be

elaborated by platelets that become activated within the tumor.

Further growth factors or ligands contemplated to be useful as tumor vasculature-associated targets include EGF, FGF, VEGF, TGF, HGF (NaKamura, 1991), angiotropin, TGF-a, TNF-a, PD-ECGF and TIE binding ligands (Bicknell and Harris, 1992). The currently preferred targets are VEGF/VPF, the FGF family of proteins, transforming growth factors (TGF-P); TGF-a; tumor necrosis factors (TNF-); angiotropin; platelet-derived endothelial cell growth factor (PD-ECGF); TIE binding ligands; pleiotropin.

Another aspect of the present invention is the use of targeting antibodies, or binding regions therefrom, that are specific for epitopes present only on ligandreceptor complexes, which epitopes are absent from both the individual (free) ligand and the receptor in its unbound form. These antibodies recognize and bind to the unique conformation that results when a ligand, such as a growth factor, binds to its receptor, such as a growth factor receptor, to form a specifically bound complex.

Such epitopes are not present on the uncomplexed forms of the ligands or receptors.

The inventors contemplate that the ligand-receptor complexes to which these antibodies bind are present in significantly higher number on tumor-associated endothelial cells than on non-tumor associated endothelial cells. Such antibodies will therefore be useful as targeting agents and will serve to further increase the specificity of the bispecific coagulants of the invention.

(i) Receptor Constructs Soluble binding domains of endothelial cell surface receptors are also contemplated for use as targeting ligands in the present invention. This concept is generally based upon the well-known sandwich binding phenomena that has been exploited in a variety of in vitro and in vivo binding protocols. Basically, as the endothelial cells express specific receptors, the cells bind to and adsorb the corresponding ligands, the ligands are then available for binding to further receptor constructs should they be introduced into the system.

A range of useful endothelial cell receptors has been identified in the foregoing sections, with VEGF/VPF, FGF, TGFP, TIE-1 and TIE-2 being particularly preferred targets. Each of these receptors could be manipulated to form a soluble binding domain for use as a targeting ligand.

4. Disease-Associated Stromal Cell Targets (a) Extracellular Matrix/Stromal Targets The usefulness of the basement membrane markers in tumoral pathology was described by Birembaut et al.

(1985). These studies showed that the distribution of basement membrane (BM) markers, type IV collagen, laminin (LM), heparan sulphate proteoglycan (HSP) and fibronectin (FN) were disrupted in tumoral pathology. Burtin et. al.

(1983) also described alterations of the basement membrane and connective tissue antigens in human metastatic lymph nodes.

A preferred target for use with the invention is RIBS. Ugarova et al. (1993) reported that conformational changes occur in fibrinogen and are elicited by its interaction with the platelet membrane glycoprotein GPIIb-IIIa. The binding of fibrinogen to membrane glycoprotein GPIIb-IIIa on activated platelets leads to platelet aggregation. This interaction results in conformational changes in fibrinogen as evidenced by the expression of receptor-induced binding sites, RIBS, epitopes which are expressed by the bound but not the free ligand.

Two RIBS epitopes have been localized by Ugarova et al. (1993). One sequence resides at 8112-119 and is recognized by MAb 9F9; the second is the RGDF sequence at Aa 95-98 and is recognized by mAb 155B16. These epitopes are also exposed by adsorption of fibrinogen onto a plastic surface and digestion of the molecule by plasmin.

Proteolytic exposure of the epitopes coincides with cleavage of the carboxyl-terminal aspects of the Aot-chains to form fragment X2. The inaccessibility of the RGDF sequence at Aa 95-98 in fibrinogen suggests that this sequence does not participate in the initial binding of the molecule to GPIIb-IIIa.

Binding of fibrinogen to its receptor alters the conformation of the carboxyl-terminal aspects of the Aachains, exposing the sequences which reside in the coiled-coil connector segments between the D and E domains

of the molecule, generating the RIBS epitopes.

In practical terms, the RIBS sequences are proposed as epitopes for use in targeting with a coaguligand. The MAbs 9F9 and 155B16 may thus be advantageously used, as may the antibodies described by Zamarron et al. (1991).

(b) Additional Cellular Targets The present invention has the further advantage that it may be used to direct coagulants to disease-associated vasculature by targeting them to cell types found within the disease region.

Platelets participate in hemostasis and thrombosis by adhering to injured blood vessel walls and accumulating at the site of injury. Although platelet deposition at sites of blood vessel injury is responsible for the primary arrest of bleeding under physiologic conditions, it can lead to vascular occlusion with ensuing ischemic tissue damage and thrombus embolization under pathologic conditions.

Interactions of platelets with their environment and with each other represent complex processes that are initiated at the cell surface. The surface membrane, therefore, provides a reactive interface between the external medium, including components of the blood vessel wall and plasma, and the platelet interior.

p-155, a multimeric platelet protein that is expressed on activated platelets (Hayward et al., 1991), may be targeted using the invention. Platelets respond to a large number of stimuli by undergoing complex biochemical. and morphological changes. These changes are involved in physiological processes including adhesion, aggregation, and coagulation. Platelet activation produces membrane alterations that can be recognized by monoclonal antibodies. The monoclonal antibody JS-1 (Hayward et al., 1991) is one such antibody contemplated for use as part of a coaguligand.

Ligand-induced binding sites (LIBS) are sites expressed on cell surface receptors only after ligand binding causes the receptor to change shape, mediate subsequent biological events. These may be seen as counterparts to RIBS and are also preferred targets for use with the present invention.

13 anti-LIBS antibodies have been developed by Frelinger et. a). (1990; 1991), any one of which may be used to deliver a coagulant to a disease or tumor site in accordance herewith. The murine monoclonal antiplatelet antibodies MA-TSPI-1 (directed against human thrombospondin) and MA-PMI-2, MA-PMI-1, and MA-LIBS-1 (directed against LIBS on human platelet glycoprotein IIb/IIIa) of Dewerchin et al. (1991) may also be used, as may RUU 2.41 and LIBS-1 of Heynen et al. (1994); OP-G2 of Tomiyama et al. (1992); and Ab-15.

Many other targets, such as antigens on smooth muscle cells, pericytes, fibroblasts, macrophages and infiltrating lymphocytes and leukocytes may also be used.

B. Coagulating Agents The second arm or element of the bispecific agents of the invention will be a component that is capable of promoting coagulation. "Coagulation promoting agents" may be coagulation factors, factors that indirectly stimulate coagulation, or they may be in the form of a second binding region that is capable of binding and releasing a coagulation factor or factor that indirectly stimulates coagulation.

1. Coagulation Factors A variety of coagulation factors may be used in connection with the present invention, as exemplified by the agents set forth below. Where a coagulation factor is covalently linked to a first binding agent, a site distinct from its functional coagulating site is used to join the molecules. Appropriate joining regions distinct from the active sites, or functional regions, of the coagulation factors are also described in each of the following sections.

(a) Tissue Factor Tissue factor (TF) is one agent capable of initiating blood coagulation. TF is the activator of the extrinsic pathway of blood coagulation and is not in direct contact with the blood under physiologically normal conditions (Osterud et al., 1986; Nemerson, 1988; Broze, 1992; Ruf & Edington, 1994). In vascular damage or activation by certain cytokines or endotoxin, however, TF will be exposed to the blood, either by the (sub)endothelial cells (Weiss et awl., 1989) or by certain blood cells (Warr et al., 1990). TF will then complex with factor VIIa, which under normal conditions circulates at low concentrations in the blood (Wildgoose et al., 1992), and the TF/factor VIIa complex will start the coagulation cascade through the activation of factor X into factor Xa. The cascade will ultimately result in the formation of fibrin.

For this sequence of events to occur, the TF:VIIa complex has to be associated with a phospholipid surface upon which the coagulation-initiation complexes with factors IX or X can assemble (Ruf et awl., 19.91; Paborsky et al., 1991; Bach et al., 1986). For this reason, truncated TF (or tTF), from which the transmembrane and cytoplasmic regions have been removed by truncating the gene, is a soluble protein having one hundred-thousandth of the factor X-activating activity of native TF (Ruf et al., 1991).

(b) Clotting Factors Thrombin, Factor V/Va and derivatives, Factor VIII/VIIIa and derivatives, Factor IX/IXa and derivatives, Factor X/Xa and derivatives, Factor XI/XIa and derivatives, Factor XII/XIIa and derivatives, Factor XIII/XIIIa and derivatives, Factor X activator and Factor V activator may also be used in the present invention.

(c) Venom Coagulants Russell's viper venom was shown to contain a coagulant protein by Williams and Esnouf in 1962. Kisiel (1979) isolated a venom glycoprotein that activates Factor V; and Di Scipio et al. (1977) showed that a protease from the venom activates human Factor X. The Factor X activator is the component contemplated for use in this invention.

Monoclonal antibodies specific for the Factor X activator present in Russell's viper venom have also been produced (e.g., MP1 of Pukrittayakamee eta)., 1983), and could be used to deliver the agent to a specific target site within the body.

(d) Prostaglandins and Synthetic Enzymes Thromboxane A2 is formed from endoperoxides by the sequential actions of the enzymes cyclooxygenase and thromboxane synthetase in platelet microsomes.

Thromboxane A2 is readily generated by platelets and is a potent vasoconstrictor, by virtue of its capacity to produce platelet aggregation (Whittle et al., 1981).

Both thromboxane A2 and active analogues thereof are contemplated for use in the present invention. A synthetic protocol for generating thromboxane A2 is described by Bhagwat et al. (1985). The thromboxane A2 analogues described by Ohuchida et. al. (1981) (especially compound 2) are particularly contemplated for use herewith.

It is possible that thromboxane synthase, and other enzymes that synthesize platelet-activating prostaglandins, may also be used as "coagulants" in the present context. Shen and Tai (1986a;b) describe monoclonal antibodies to, and immunoaffinity purification of, thromboxane synthase; and Wang et. a). (1991) report the cDNA for human thromboxane synthase.

(e) Inhibitors of Fibrinolysis a2-antiplasmin, or a2-plasmin inhibitor, is a proteinase inhibitor naturally present in human plasma that functions to efficiently inhibit the lysis of fibrin clots induced by plasminogen activator (Moroi & Aoki, 1976). a2-antiplasmin is a particularly potent inhibitor, and is contemplated for use in the present invention.

a2-antiplasmin may be purified as first described by Moroi and Aoki (1976). Other purification schemes are also available, such as using affinity chromatography on plasminogen-Sepharose, ion-exchange chromatography on DEAE-Sephadex and chromatography on Concanavalin-A Sepharose; or using affinity chromatography on a Sepharose column bearing an elastase-digested plasminogen formulation containing the three N-terminal triple-loop structures in the plasmin A-chain (LBSI), followed by gel filtration (Wiman & Collen, 1977; Wiman, 1980, respectively).

As the cDNA sequence for a2-antiplasmin is available (Tone et al., 1977), a preferred method for a2-antiplasmin production will be via recombinant expression.

Monoclonal antibodies against a2-antiplasmin are also available that may be used in the bispecific binding ligand embodiments of the invention. For example, Hattey et al. (1987) described two MAbs against a2-antiplasmin, MPW2AP and MPW3AP. As each of these MAbs were reported to react equally well with native a2-antiplasmin, they could both be used to deliver exogenous a2-antiplasmin to a target site or to garner endogenous a2-antiplasmin and concentrate it within the targeted region. Other antibodies, such as JTPI-2, described by Mimuro and colleagues, could also be used.

2. Agents that Bind Coagulation Factors Another group of bispecific coagulating ligands of this invention are those in which the targeting region is not directly linked to a coagulation factor, but is linked to a second

binding region that binds to a coagulating factor.

Where a second binding region is used to bind and deliver a coagulation factor, the binding region is chosen so that it recognizes a site on the coagulation factor that does not significantly impair its ability to induce coagulation. The regions of the coagulation factors suitable for binding in this manner will generally be the same as those regions that are suitable for covalent linking to the targeting region, as described in the previous sections.

However, in that bispecific ligands of this class may be expected to release the coagulation factor following delivery to the tumor site or region, there is more flexibility allowed in the regions of the coagulation factor suitable for binding to a second binding agent or antibody. Another advantage is that bispecific antibodies can be pre-localized before infusion of tTF which may reduce the amount of tTf required and hence toxicity.

Suitable second binding regions for use in this manner, will generally be antigen combining sites of antibodies that have binding specificity for the coagulation factor, including functional portions of antibodies, such as scFv, Fv, Fab', Fab and F(ab')2 fragments.

Bispecific binding ligands that contain antibodies, or fragments thereof, directed against Tissue Factor, Thrombin, Prekallikein, Factor V/Va, Factor VIII/VIIIa, Factor IX/IXa, Factor X/Xa, Factor XI/XIa, Factor XII/XIIa, Factor XIII/XIIIa, Russell's viper venom, thromboxane A2 or a2-antiplasmin are exemplary embodiments of this aspect of the invention.

C. Linkage Means The first, targeting region and second, coagulating region will be operatively linked to allow each region to perform its intended function without significant impairment. Thus, the targeting region is capable of binding to the intended target, as selected from the range of tumor environment targets, and the coagulating region is capable of directly or indirectly, e.g., through the release of a bound factor, promoting blood coagulation or clotting.

To assess the targeting region binding function, all that is required is to conduct a binding assay to ensure that the bispecific ligand still binds to the targeted component in substantially the same manner as the uncomplexed first binding region. The suitable binding assays are of the type usually seen in immunological binding assays, where the first targeting region is an antibody, and/or other biochemical binding assays, e.g., those using 125Iodine labeled proteins or other radiolabeled components, as used to assess ligandreceptor binding, to generate Scatchard plots, and the like.

The target antigen or component in such assays may be provided in many forms, including proteins purified from natural or recombinant sources, membrane enriched preparations, intact cells and tissue sections.

Generally, where protein compositions are used, they will immobilized on a solid support, such as a microtitre plate, a membrane, or even on a column matrix. It is also generally preferred to use a target composition that reflects the physiological target, therefore as the target will usually be cell-associated, the use of compositions that include intact cells, including tissues and the cells themselves, is also preferred.

The various immunological assays available to confirm the functional binding of a bispecific complex include, e.g., Western blots, ELISAs, ELISAs using fixed cells, immunohistochemistry, and fluorescent activated cell sorting (FACS). The execution of all such assays is generally known to those of skill in the art, and is further disclosed herein.

Assessing the targeting region binding function of a bispecific compound in any of the above or other binding assays is a straightforward matter, where the bispecific ligand and the uncomplexed first binding region will most usually be run in a parallel assay, under the same conditions, to enable ready comparison. Effective bispecific ligands will bind to the target without significant impairment, i.e., in substantially the same manner as the uncomplexed first binding region. Taking the uncomplexed binding region assay result as the 100% reference value, "substantial binding" of the bispecific ligand, as used herein, means that the bispecific ligand exhibits at least about 50% binding, and more preferably, between about 50% and about 80k binding, and most preferably, between about 80 and about 100t binding.

Where the bispecific ligand includes a second binding region that binds to a coagulant, e.g., it is a bispecific antibody, further useful assays are those of the type that allow the binding functions of both arms of the bispecific ligand to be assessed at the same time.

For example, this may be achieved by assessing the binding of a radiolabeled coagulant to a target cell via bridging with the bispecific ligand or antibody. Such an assay is exemplified by the binding of tTF to target cells using the B21-2/10H10 bispecific antibody, as described in Example II.

Determining the coagulating agent function of the bispecific ligand is also a straightforward matter. All that is required here is to conduct a coagulation assay~ using the bispecific ligand and ensure that it functions to promote coagulation in substantially the same manner as the uncomplexed coagulating agent. This is true for "coagulating agents" that are both coagulation factors themselves and those that are second binding regions that bind to a coagulation factor. Naturally, in the latter case, in an in vitro or ex vivo assay, the bispecific ligand will be precomplexed with the coagulation factor to allow binding to the second binding region.

One suitable coagulation assay is that in which the bispecific ligands, pre-complexed with coagulant if necessary, are admixed with a plasma sample. The appearance of fibrin strands is indicative of coagulation in this assay. Effective bispecific ligands would thus be expected to reduce the time taken for fibrin strands to appear, and particularly, to significantly reduce the elapsed time in comparison to control levels.

A variation of the above assay involves first exposing appropriate target cells to the bispecific ligand under conditions effective, and for a time sufficient, to allow binding, washing the cells to remove non-specifically bound components and then resuspending the washed cells in plasma. Only cells effectively coated with the bispecific ligand would be expected to reduce the time taken for fibrin strands to appear in the assay. This type of assay is preferred in that it is, in itself, an assay that assesses both of the functions of the bispecific construct, i.e., initial targeting to the cell and subsequent localized coagulation.

To compare the coagulating function of a bispecific compound to that of an uncomplexed coagulating agent, parallel assays may again be conducted. Effective bispecific ligands will function to promote coagulation without significant impairment, i.e., will function in substantially the same manner as the uncomplexed coagulating agent. Taking the uncomplexed coagulant assay result as the 100% reference value, "substantial function", as used herein, means that the bispecific ligand exhibits at least about 50% coagulation, and more preferably, between about 50% and about 80% coagulation, and most preferably, between about 80% and about 100% coagulation.

The two functional regions of the bispcific ligands may be joined using synthetic chemistry techniques or recombinant DNA techniques. Each of these techniques are routinely employed and well known to those of skill in the art, and are further exemplified in Example I and by the details set forth below.

1. Biochemical Cross-linkers The joining of an antibody, or other targeting component, to a coagulating agent will generally employ the same technology as developed for the preparation of immunotoxins. However, considerable advantages are apparent in the present technology, as the consequences of a certain amount of uncomplexed coagulating agent becoming available physiologically are not contemplated to be particularly severe. Thus, the stability requirements for any cross-linkers are not so stringent as for linkers employed in other constructs, such as immunotoxins. Therefore, it can be considered as a general guideline that any biochemical crosslinker that is appropriate for use in an immunotoxin will also be of use in the present context, and additional linkers may also be considered.

In addition to toxins, a variety of other chemotherapeutic and pharmacological agents have been linked to antibodies to form conjugates that have been shown to function pharmacologically (see, e.g., Vaickus eta)., 1991). Exemplary antineoplastic agents that have been investigated include doxorubicin, daunomycin, methotrexate and vinblastine, amongst others (Dillman eta)., 1988; Pietersz eta)., 1988). Moreover, the attachment of other agents such as neocarzinostatin (Kimura et awl., 1983), macromycin (Manabe et awl., 1984), trenimon (Ghose, 1982) and a-amanitin (Davis & Preston, 1981) has been described. The linking technology described in each of the foregoing scientific papers is also contemplated for use in connection with the present invention.

Cross-linking reagents are used to form molecular bridges that tie together functional groups of two different molecules, e.g., a binding and coagulating agent. To link two different proteins in a step-wise manner, heterobifunctional cross-linkers can be used that eliminate unwanted homopolymer formation (Table VI).
TABLE VI HETEROBIFUNCTIONAL CROSS-LINKERS EMI107.1

Spacer Arm
Length\after
linker Reactive Toward Advantages and Applications cross-linking
SMPT Primary amines . Greater stability 11.2
Sulfhydryls
SPDP Primary amines . Thiolation 6.8
Sulfhydryls . Cleavable cross-linking
LC-SPDP Primary amines . Extended spacer arm 15.6
Sulfhydryls
Sulfo-LC- Primary amines . Extended spacer arm 15.6
SPDP Sulfhydryls . Water-soluble
SMCC Primary amines . Stable maleimide reactive 11.6
Sulfhydryls group
. Enzyme-antibody conjugation
. Hapten-carrier protein
conjugation
Sulfo-SMCC Primary amines . Stable maleimide reactive 11.6
Sulfhydryls group
. Water-soluble
. Enzyme-antibody conjugation
MBS Primary amines . Enzyme-antibody conjugation 9.9
Sulfhydryls . Hapten-carrier protein
conjugation
Sulfo-MBS Primary amines . Water-soluble 9.9
Sulfhydryls
TABLE VI continued... EMI108.1

Spacer Arm
Length\after
linker Reactive Toward Advantages and Applications cross-linking
SIAB Primary amines . Enzyme-antibody conjugation 10.6
Sulfhydryls
Sulfo-SIAB Primary amines . Water-soluble 10.6
Sulfhydryls
SMPB Primary amines . Extended spacer arm 14.5
Sulfhydryls . Enzyme-antibody conjugation
Sulfo-SMPB Primary amines . Extended spacer arm 14.5
Sulfhydryls . Water-soluble
EDC/Sulfo- Primary amines . Hapten-Carrier conjugation 0
NHS Carboxyl groups
ABH Carbohydrates . Reacts with sugar groups 11.9
Nonselective

An exemplary heterobifunctional cross-linker contains two reactive groups: one reacting with primary amine group (e.g., N-hydroxy succinimide) and the other reacting with a thiol group (e.g., pyridyl disulfide, maleimides, halogens, etc.). Through the primary amine reactive group, the cross-linker may react with the lysine residue(s) of one protein (e.g., the selected antibody or fragment) and through the thiol reactive group, the cross-linker, already tied up to the first protein, reacts with the cysteine residue (free sulfhydryl group) of the other protein (e.g., the coagulant).

It can therefore be seen that the preferred coagulants or coagulant binding regions will generally have, or be derivatized to have, a functional group available for cross-linking purposes. This requirement is not considered to be limiting in that a wide variety of groups can be used in this manner. For example, primary or secondary amine groups, hydrazide or hydrazine groups, carboxyl alcohol, phosphate, or alkylating groups may be used for binding or cross-linking. For a general overview of linking technology, one may wish to refer to Ghose & Blair (1987).

The spacer arm between the two reactive groups of a cross-linkers may have various length and chemical compositions. A longer spacer arm allows a better flexibility of the conjugate components while some particular components in the bridge (e.g., benzene group) may lend extra stability to the reactive group or an increased resistance of the chemical link to the action of various aspects (e.g., disulfide bond resistant to

reducing agents). The use of peptide spacers, such as L-Leu-L-Ala-L-Leu-L-Ala, is also contemplated.

It is preferred that a cross-linker having reasonable stability in blood will be employed. Numerous types of disulfide-bond containing linkers are known that can be successfully employed to conjugate targeting and coagulating agents. Linkers that contain a disulfide bond that is sterically hindered may prove to give greater stability in vivo, preventing release of the coagulant prior to binding at the site of action. These linkers are thus one preferred group of linking agents.

One of the most preferred cross-linking reagents for use in immunotoxins is SMPT, which is a bifunctional cross-linker containing a disulfide bond that is "sterically hindered11 by an adjacent benzene ring and methyl groups. It is believed that stearic hindrance of the disulfide bond serves a function of protecting the bond from attack by thiolate anions such as glutathione which can be present in tissues and blood, and thereby help in preventing decoupling of the conjugate prior to the delivery of the attached agent to the tumor site. It is contemplated that the SMPT agent may also be used in connection with the bispecific coagulating ligands of this invention.

The SMPT cross-linking reagent, as with many other known cross-linking reagents, lends the ability to crosslink functional groups such as the SH of cysteine or primary amines (e.g., the epsilon amino group of lysine).

Another possible type of cross-linker includes the heterobifunctional photoreactive phenylazides containing a cleavable disulfide bond such as sulfosuccinimidyl-2 (p-azido salicylamido) ethyl-1,3'-dithiopropionate. The N-hydroxy-succinimidyl group reacts with primary amino groups and the phenylazide (upon photolysis) reacts nonselectively with any amino acid residue.

In addition to hindered cross-linkers, non-hindered linkers can also be employed in accordance herewith.

Other useful cross-linkers, not considered to contain or generate a protected disulfide, include SATA, SPDP and 2iminothiolane (Wawrzynczak & Thorpe, 1987). The use of such cross-linkers is well understood in the art.

Once conjugated, the bispecific agent will generally be purified to separate the conjugate from unconjugated targeting agents or coagulants and from other contaminants. It is important to remove unconjugated targeting agent to avoid the possibility of competition for the antigen between conjugated and unconjugated species. A large a number of purification techniques are available for use in providing conjugates of a sufficient degree of purity to render them clinically useful.

Purification methods based upon size separation, such as gel filtration, gel permeation or high performance liquid chromatography, will generally be of most use. Other chromatographic techniques, such as Blue-Sepharose separation, may also be used.

2. Recombinant Fusion Proteins The bispecific targeted coagulants of the invention may also be fusion proteins prepared by molecular biological techniques. The use of recombinant DNA techniques to achieve such ends is now standard practice to those of skill in the art. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. DNA and RNA synthesis may, additionally, be performed using an automated synthesizers (see, for example, the techniques described in Sambrook et al., 1989; and Ausubel et al., 1989).

In general, to prepare a fusion a protein one would join a DNA coding region, such as a gene or cDNA, encoding a binding ligand or other targeting region to a DNA coding region (i.e., gene or cDNA) encoding a coagulation factor or coagulant binding region. This typically involves preparing an expression vector that comprises, in the same reading frame, a first DNA segment encoding the first binding region operatively linked to a second DNA segment encoding the coagulation factor. The sequences are attached -in a manner such that translation of the total nucleic acid yields the desired bispecific compounds of the invention. Expression vectors contain one or more promoters upstream of the inserted DNA regions that act to promote transcription of the DNA and to thus promote expression of the encoded recombinant protein. This is the meaning of "recombinant expression".

Should a particular binding region or coagulant be preferred, and the encoding DNA not instantly available, it may be obtained using the techniques of "molecular cloning" in which a DNA molecule encoding the

desired protein is obtained from a DNA library (e.g., a cDNA or genomic library). In such procedures, an appropriate DNA library is screened, e.g., using an expression screening protocol employing antibodies directed against the protein, or activity assays. Alternatively, screening may be based on the hybridization of oligonucleotide probes, designed from a consideration of portions of the amino acid sequence of the protein, or from the DNA sequences of genes encoding related proteins. The operation of such screening protocols are well known to those of skill in the art and are described in detail in the scientific literature, for example, in Sambrook et al. (1989).

When produced via recombinant DNA techniques, the targeting agent/coagulating agent compounds of the invention are referred to as "fusion proteins". It is to be understood that such fusion proteins contain, at least, a targeting agent and a coagulating agent as defined in this invention, and that the agents are operatively attached. The fusion proteins may also include additional peptide sequences, such as peptide spacers which operatively attach the targeting agent and coagulating agent compounds, as long as such additional sequences do not appreciably affect the targeting or coagulating activities of the resultant fusion protein.

It will be understood that the recombinant bispecific protein ligands may differ from those bispecific constructs generated by chemically crosslinking the so-called naturally-produced proteins. In particular, the degree of post-translational modifications, such as, for example, glycosylation and phosphorylation may be different between recombinant fusions and chemical fusions of the same two proteins.

This is not contemplated to be a significant problem, however, those of skill in the art will know to confirm that a recombinant fusion protein functions as intended, and expected from other data, before use in a clinical setting.

One advantage of recombinant expression is that the linking regions can be readily manipulated so that, e.g., their length and/or amino acid composition is readily variable. Non-cleavable peptide spacers may be provided to operatively attach the two agents of the invention, if desired. Equally, peptides with unique cleavage sites could be inserted between the two components.

If desired in a specific instance, it is possible to provide a peptide spacer operatively attaching the targeting agent and coagulating agent which is capable of folding into a disulfide-bonded loop structure.

Proteolytic cleavage within the loop would then yield a heterodimeric polypeptide wherein the targeting agent and the coagulating agent are linked by only a single disulfide bond (see, for example, Lord et al., 1992).

Many standard techniques are available to construct expression vectors containing the appropriate nucleic acids and transcriptional/ translational control sequences in order to achieve protein expression in a variety of host-expression systems. The cell types available for expression include, but are not limited to, microorganisms such as bacteria (e.g., E. coli, B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing targeting agent/coagulant coding sequences; yeast (e.g., Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing targeting agent/coagulating agent coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the targeting agent/coagulating agent coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the targeting agent/coagulant coding sequence; and mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the targeting agent/coagulating agent construct being expressed. For example, when large quantities of bispecific agent are to be produced, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable.Such vectors include, but are not limited to, the E. coli expression vector pUR278 (Ruther et al., 1983), in which the targeting agent/coagulating agent coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein additionally containing a portion of the lac Z product is provided; pIN vectors (Inouye et al., 1985; Van Heeke et al., 1989); and the like. pGEX vectors may also be used to express foreign

polypeptides, such as the targeting agent/coagulating agent combinations as fusion proteins additionally containing glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione.

The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the binding agent/coagulant protein of the overall fusion protein can be released from the GST moiety.

In a useful insect system, Autograph californica nuclear polyhidrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The targeting agent/coagulating agent coding sequences may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the bispecific ligand coding sequences will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene).

These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed (e.g., see Smith et al., 1983; *U.S. Patent No.

4,215,051, Smith) - In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the targeting agent/coagulating agent coding sequences may be ligated to an adenovirus transcription/ translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination.

Insertion in a non-essential region of the viral genome (e.g., region El or E3) will result in a recombinant virus that is viable and capable of expressing bispecific proteins in infected hosts (e.g., see Logan et al., 1984) Specific initiation signals may also be required for efficient translation of inserted targeting agent/coagulating agent coding sequences. These signals include the ATG initiation codon and adjacent sequences.

Exogenous translational control signals, including the ATG initiation codon, may additionally need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be in phase (or in-frame) with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic.

The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987) .

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express constructs encoding the targeting agent/coagulant ligands may be engineered.

Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with targeting agent/coagulant DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker.

Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their

chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

A number of selection systems may be used, including, but not limited, to the herpes simplex virus thymidine kinase (Wigler et al., 1977), hypoxanthineguanine phosphoribosyltransferase (Szybalska et al., 1962), and adenine phosphoribosyltransferase genes (Lowy et al., 1980) can be employed in tk-, hgprt- or aprtcells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., 1980; O'Hare et al., 1981); gpt, which confers resistance to mycophenolic acid (Mulligan et al., 1981); neo, which confers resistance to the aminoglycoside G-418 (Colberre Garapin et al., 1981); and hygro, which confers resistance to hygromycin (Santerre et al., 1984).

D. Antibodies Where antibodies are used as one or both portions of the bispecific ligand, the choice of antibody will generally be dependent on the type tumor and coagulating ligand chosen. However, certain advantages may be achieved through the application of particular types of antibodies. For example, while IgG based antibodies may be expected to exhibit better binding capability and slower blood clearance than their Fab' counterparts, Fab' fragment-based compositions will generally exhibit better tissue penetrating capability.

1. Monoclonal Antibodies Means for preparing and characterizing antibodies are well known in the art (See, e.g., *Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic composition in accordance with the present invention, either with or without prior immunotolerizing, depending on the antigen composition and protocol being employed (e.g., tolerizing to a normal cell population and then immunizing with a tumor cell population), and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimyde and bis-biazotized benzidine.

As is also well known'in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a nonspecific stimulator of the immune response containing killed Mycobacterium tuberculosis), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired titer level is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

MAbs may be readily prepared through use of wellknown techniques, such as those exemplified in *U.S.

Patent 4,196,265, incorporated herein by reference.

Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, e.g., a purified or partially purified tumor cell or vascular endothelial cell protein, polypeptide, peptide, or intact cell composition. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit,

sheep frog cells is also possible. The use of rats may provide certain advantages (*Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5 X 107 to 2 X 108 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (*Goding, pp.

65-66, 1986; *Campbell, pp. 75-83, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F, 4B210 or one of the above listed mouse cell lines; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6, are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the A63-A68, 653 myeloma cell line, which is readily available from the ATCC. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 4:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler & Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter et al. (1977). The use of electrically induced fusion methods is also appropriate (*Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1 X 10-6 to 1 X 10-8. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the de novo synthesis of nucleotides in the tissue culture media.

Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block de novo synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium) - Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, e.g., hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired

reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration.

The individual cell lines could also be cultured in vitro, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

The inventors also contemplate the use of a molecular cloning approach to generate monoclonals. For this, combinatorial immunoglobulin phagemid libraries are prepared from RNA isolated from the spleen of the immunized animal, and phagemids expressing appropriate antibodies are selected by panning using cells expressing the antigen and control cells e.g., normal-versus-tumor cells. The advantages of this approach over conventional hybridoma techniques are that approximately 104 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H and L chain combination which further increases the chance of finding appropriate antibodies.

Where MAbs are employed in the present invention, they may be of human, murine, monkey, rat, hamster, chicken or even rabbit origin. The invention contemplates the use of human antibodies, "humanized" or chimeric antibodies from mouse, rat, or other species, bearing human constant and/or variable region domains, and other recombinant antibodies and fragments thereof.

Of course, due to the ease of preparation and ready availability of reagents, murine monoclonal antibodies will typically be preferred.

2. Functional Antibody Binding Regions The origin or derivation of the targeting agent antibody or antibody fragment (e.g., Fab', Fab, F(ab')2, Fv or scFv) is not believed to be particularly crucial to the practice of the invention, so long as the antibody or fragment that is actually employed for the preparation of the bispecific ligand exhibits the desired binding properties.

It may be necessary to use antibody preparations in which the Fc portion has been removed. Fragmentation of immunoglobulin molecules can be achieved by controlled proteolysis, although the conditions will vary considerably with species and immunoglobulin class or subclass. Bivalent F(ab)2 fragments are usually preferable over the univalent Fab or Fab fragments.

Fab Fab fragments can be obtained by proteolysis of the whole immunoglobulin by the non-specific thiol protease, papain. Papain must first be activated by reducing the sulphydryl group in the active site with cysteine, 2mercaptoethanol or dithiothreitol. Heavy metals in the stock enzyme should be removed by chelation with EDTA (2 mM) to ensure maximum enzyme activity. Enzyme and substrate are normally mixed together in the ratio of 1:100 by weight. After incubation, the reaction can be stopped by irreversible alkylation of the thiol group with iodoacetamide or simply by dialysis. The completeness of the digestion should be monitored by SDS PAGE and the various fractions separated by protein A Sepharose or ion exchange chromatography.

F(ab')2 The usual procedure for preparation of F(ab')2 fragments from IgG of rabbit and human origin is limited proteolysis by the enzyme pepsin (Protocol 7.3.2). The conditions, IOOx antibody excess w/w in acetate buffer at pH 4.5, 370C, suggest that antibody is cleaved at the Cterminal side of the inter-heavy-chain disulfide bond.

Rates of digestion of mouse IgG may vary with subclass and it may be difficult to obtain high yields of active F(ab')2 fragments without some undigested or completely degraded IgG. In particular, IgG2b is highly susceptible to complete degradation. The other subclasses require different incubation conditions to produce optimal results.

Digestion of rat IgG by pepsin requires conditions including dialysis in 0.1 M acetate buffer, pH 4.5, and then incubation for four hours with 18 w/w pepsin; IgG1 and IgG2a digestion is improved if first dialysed against 0.1 M formate buffer, pH 2.8, at 40C, for 16 hours followed by acetate buffer. IgG2 gives more consistent results with incubation in staphylococcal V8 protease (3 w/w) in 0.1 M sodium phosphate buffer, pH 7.8, for four hours at 370C.

3. Bispecific Antibodies In general, the preparation of bispecific antibodies is also well known in the art, as exemplified by Glennie et al. (1987). Bispecific antibodies have been employed clinically, for example, to treat cancer patients (Bauer et al., 1991). One method for the preparation of bispecific antibodies involves the separate preparation of antibodies having specificity for the targeted tumor cell antigen, on the one hand, and the coagulating agent (or other desired target, such as an activating antigen) on the other.

Bispecific antibodies have also been developed particularly for use as immunotherapeutic agents. As mentioned earlier in conjunction with antigen-induction, certain of these antibodies were developed to cross-link lymphocytes and tumor antigens (Nelson, 1991; Segal et al., 1992). Examples include chimeric molecules that bind T cells, e.g., at CD3, and tumor antigens, and trigger lymphocyte-activation by physically cross-linking the TCR/CD3 complex in close proximity to the target cell (Staerz et al., 1985; Perez et al., 1985; 1986a; 1986b; Ting et al., 1988).

Indeed, tumor cells of carcinomas, lymphomas, leukemias and melanomas have been reported to be susceptible to bispecific antibody-mediated killing by T cells (Nelson, 1991; Segal et al., 1992; deLeij et al., 1991). These type of bispecific antibodies have also been used in several Phase I clinical trials against diverse tumor targets. Although they are not novel compositions in accordance with this invention, the combined use of bispecific cross-linking antibodies along with the bispecific coagulating ligands described herein is also contemplated. The bispecific cross-linking antibodies may be administered as described in references such as deLeij et al. (1991); Clark et al. (1991); Rivoltini et al. (1992); Bolhuis et al. (1992); and Nitta et al. (1990).

While numerous methods are known in the art for the preparation of bispecific antibodies, the Glennie et al.

(1987) method involves the preparation of peptic F(ab'Y)2 fragments from the two chosen antibodies, followed by reduction of each to provide separate Fab'ysH fragments.

The SH groups on one of the two partners to be coupled are then alkylated with a cross-linking reagent such as o-phenylenedimaleimide to provide free maleimide groups on one partner. This partner may then be conjugated to the other by means of a thioether linkage, to give the desired F (abt) 2 heteroconjugate.

Due to ease of preparation, high yield and reproducibility, the Glennie et al. (1987) method is often preferred for the preparation of bispecific antibodies, however, there are numerous other approaches that can be employed and that are envisioned by the inventors. For example, other techniques are known wherein crosslinking with SPDP or protein A is carried out, or a trispecific construct is prepared (Titus et al., 1987; Tutt et al., 1991).

Another method for producing bispecific antibodies is by the fusion of two hybridomas to form a quadroma (Flavell et al., 1991, 1992; Pimm et al., 1992; French et al., 1991; Embleton et awl., 1991). As used herein, the term "quadroma" is used to describe the productive fusion of two B cell hybridomas. Using now standard techniques, two antibody producing hybridomas are fused to give daughter cells, and those cells that have maintained the expression of both sets of clonotype immunoglobulin genes are then selected.

A preferred method of generating a quadroma involves the selection of an enzyme deficient mutant of at least one of the parental hybridomas. This first mutant hybridoma cell line is then fused to cells of a second hybridoma that had been lethally exposed, e.g., to iodoacetamide, precluding its continued survival. Cell fusion allows for the rescue of the first hybridoma by acquiring the gene for its enzyme deficiency from the lethally treated hybridoma, and the rescue of the second hybridoma through fusion to the first hybridoma.

Preferred, but not required, is the fusion of immunoglobulins of the same isotype, but of a different subclass. A mixed subclass antibody permits the use if an alternative assay for the isolation of a preferred quadroma.

In more detail, one method of quadroma development and screening involves obtaining a hybridoma line

that secretes the first chosen MAb and making this deficient for the essential metabolic enzyme, hypoxanthine-guanine phosphoribosyltransferase (HGPRT). To obtain deficient mutants of the hybridoma, cells are grown in the presence of increasing concentrations of 8-azaguanine (1 x 10-7M to 1 x 105M). The mutants are subcloned by limiting dilution and tested for their hypoxanthine/ aminopterin/ thymidine (HAT) sensitivity. The culture medium may consist of, for example, DMEM supplemented with 10% FCS, 2 mM L-Glutamine and 1 mM penicillin-streptomycin.

A complementary hybridoma cell line that produces the second desired MAb is used to generate the quadromas by standard cell fusion techniques (Galfre et al., 1981), or by using the protocol described by Clark et al.

(1988). Briefly, 4.5 x 107 HAT-sensitive first cells are mixed with 2.8 x 107 HAT-resistant second cells that have been pre-treated with a lethal dose of the irreversible biochemical inhibitor iodoacetamide (5 mM in phosphate buffered saline) for 30 minutes on ice before fusion.

Cell fusion is induced using polyethylene glycol (PEG) and the cells are plated out in 96 well microculture plates. Quadromas are selected using HAT-containing medium. Bispecific antibody-containing cultures are identified using, for example, a solid phase isotypespecific ELISA and isotype-specific immunofluorescence staining.

In one identification embodiment to identify the bispecific antibody, the wells of microtiter plates (Falcon, Becton Dickinson Labware) are coated with a reagent that specifically interacts with one of the parent hybridoma antibodies and that lacks crossreactivity with both antibodies. The plates are washed, blocked, and the supernatants (SNs) to be tested are added to each well. Plates are incubated at room temperature for 2 hours, the supernatants discarded, the plates washed, and diluted alkaline phosphatase-antiantibody conjugate added for 2 hours at room temperature.

The plates are washed and a phosphatase substrate, e.g., P-Nitrophenyl phosphate (Sigma, St. Louis) is added to each well. Plates are incubated, 3N NaOH is added to each well to stop the reaction, and the OD410 values determined using an ELISA reader.

In another identification embodiment, microtiter plates pre-treated with poly-L-lysine are used to bind one of the target cells to each well, the cells are then fixed, e.g. using 1% glutaraldehyde, and the bispecific antibodies are tested for their ability to bind to the intact cell. In addition, FACS, immunofluorescence staining, idiotype specific antibodies, antigen binding competition assays, and other methods common in the art of antibody characterization may be used in conjunction with the present invention to identify preferred quadromas.

Following the isolation of the quadroma, the bispecific antibodies are purified away from other cell products. This may be accomplished by a variety of protein isolation procedures, known to those skilled in the art of immunoglobulin purification. Means for preparing and characterizing antibodies are well known in the art (See, e.g., *Antibodies: A Laboratory Manual, 1988) .

For example, supernatants from selected quadromas are passed over protein A or protein G sepharose columns to bind IgG (depending on the isotype). The bound antibodies are then eluted with, e.g. a pH 5.0 citrate buffer. The elute fractions containing the BsAbs, are dialyzed against an isotonic buffer. Alternatively, the eluate is also passed over an anti-immunoglobulinsepharose column. The BsAb is then eluted with 3.5 M magnesium chloride. BsAbs purified in this way are then tested for binding activity by, e.g., an isotype-specific ELISA and immunofluorescence staining assay of the target cells, as described above.

Purified BsAbs and parental antibodies may also be characterized and isolated by SDS-PAGE electrophoresis, followed by staining with silver or Coomassie. This is possible when one of the parental antibodies has a higher molecular weight than the other, wherein the band of the BsAbs migrates midway between that of the two parental antibodies. Reduction of the samples verifies the presence of heavy chains with two different apparent molecular weights.

Furthermore, recombinant technology is now available for the preparation of antibodies in general, allowing the preparation of recombinant antibody genes encoding an antibody having the desired dual specificity (Van Duk et al., 1989). Thus, after selecting the monoclonal antibodies having the most preferred binding characteristics, the respective genes for these antibodies can be isolated, e.g., by immunological screening

of a phage expression library (Oi & Morrison, 1986; Winter & Milstein, 1991). Then, through rearrangement of Fab coding domains, the appropriate chimeric construct can be readily obtained.

E. Binding Assays Although the present invention has significant utility in animal and human treatment regimens, it also has many other practical uses. These uses are generally related to the specific binding ability of the bispecific compounds. In that all the compounds of the invention include at least one targeting and binding component, e.g., an antibody, ligand, receptor, or such like, the resultant bispecific construct may be used in virtually all of the binding embodiments that the original antibody, ligand or receptor, etc., may be used. The presence of the coagulant, or other binding regions, does not negate the utility of the first binding regions in any binding assay.

As such, the bispecific coagulating ligands may be employed in standard binding assays, such as in immunoblots, Western blots, and other assays in which an antigen is immobilized onto a solid support matrix, e.g., nitrocellulose, nylon or a combination thereof. They may be employed simply as an "antibody substitute" or may be used to provide a more-specific detection means for use in detecting antigens against which standard secondary reagents cause an unacceptably high background. This is especially useful when the antigens studied are themselves immunoglobulins or other antibodies are used in the procedure, as exemplified below in the case of ELISAs.

The bispecific binding ligands may also be used in conjunction with both fresh-frozen and formalin-fixed, paraffin-embedded tissue blocks in immunohistochemistry; in fluorescent activated cell sorting, flow cytometry or flow microfluorometry; in immunoprecipitation to separate a target antigen from a complex mixture, in which case, due to their potential to form molecular lattices, they may even achieve precipitation without a secondary matrix-coupled reagent; in antigen or cell purification embodiments, such as affinity chromatography, even including, in certain cases, the one-step rapid purification of one or more cell populations at the same time; and in many other binding assays that will be known to those of skill in the art given the information presented herein.

As an example, the bispecific ligands of the invention may be used in ELISA assays. Many types of ELISAs are known and routinely practiced in the art. The bispecific ligands may be employed in any of the binding steps, depending on the particular type of ELISA being performed and the "antigen" (component) to be detected.

The ligands could therefore be used to coat the plate, to compete for binding sites, as an antigen to provide a standard curve, as a primary binding ligand, as a secondary binding ligand or even as a tertiary or other binding ligand. The many modes of conducting ELISAs will be known to those of skill in the art, in further light of the exemplary mode discussed below.

In one form of an ELISA, binding targets, generally antibodies themselves, are immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, it is desirable to bind or coat the assay plate wells with a nonspecific protein that is known to be antigenically neutral with regard to the test antisera such as bovine serum albumin (BSA), casein or solutions of milk powder. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

In these types of ELISAs, generally termed sandwich ELISAs, the plate-bound antibody is used to "trap" the antigen. After binding of the first antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with, in the present exemplary embodiment, a test sample containing the antigenic material to be detected and/or titered in a manner conducive to immune complex (antigen/antibody) formation. These embodiments are particularly useful for detecting ligands in clinical samples or biological extracts. The samples are preferably diluted with solutions of BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS) and a detergent, e.g.

Tween.

The layered antisera is then allowed to incubate for from 2 to 4 hours, at temperatures preferably on the order of 25 *to 370C. Following incubation, the antisera-contacted surface is washed so as to remove nonimmunocomplexed material. A preferred washing procedure includes washing with a solution such as

PBS/Tween, or borate buffer.

Following formation of specific immunocomplexes between the bound antigen and the test sample, and subsequent washing, the occurrence and amount of immunocomplex formation may be determined by subjecting same complex to a secondary specific binding component, which is generally an antibody-based component. In a particular embodiment, the bispecific ligands of the invention are proposed for use in this step. Further specific binding and washing steps are then conducted.

To provide a detecting means, in the present exemplary embodiment, a third antibody is used that is linked to a detectable label, such as an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate.

The third, or tertiary, labeled antibody has binding affinity for a component of the bispecific ligand. The ultimate immunocomplex is determined, after appropriate binding and washing steps, by detecting the label, e.g., by incubating with a chromogenic substrate, such as urea and bromocresol purple or 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid [ABTS] and H202.

Quantification is then achieved by measuring the degree of color generation, e.g., using a visible spectra spectrophotometer.

Using a bispecific coagulating ligand as a secondary detection reagent in conjunction with the type of ELISA described above has distinct advantages. For example, it allows the use of a tertiary, labeled antibody that is specific for a portion of the bispecific ligand that is distinct from the typical antibody constant regions usually targeted. In particular, a tertiary binding ligand that is specific for the coagulant portion (or coagulant binding region) of the bispecific construct may be employed. This novel means of detecting immune complex formation imparts improved specificity, which is particularly useful in sandwich ELISAs where the tertiary antibody may cross-react with, and bind to, the original material used to coat the plate, i.e., the original antibody, rather than just binding to the intended secondary antibody.By directing the labelled tertiary component to an non-antibody portion, or even to a novel antigen combining region, of a bispecific ligand, the problem of non-specific binding, and unusually high background, will be avoided.

Further practical uses of the bispecific ligands are evident by exploiting their coagulating ability. As all of the proposed compounds are capable of inducing coagulation, they may be employed, e.g., as a control, in any assay that involves coagulation as a component. The presence of the targeting component does not negate the utility of the coagulant in such assays, as each component functions independently of the other.

F. Effective Use of Tissue Factor-Binding Bispecific Antibodies As mentioned earlier, tissue factor (TF) is one agent capable of initiating blood coagulation. TF is exposed to the blood in vascular damage or following activation by certain cytokines. Available TF then complexes with factor VIIa to initiate the coagulation cascade that ultimately results in fibrin formation.

In one exemplary embodiment, the inventors have synthesized a bispecific antibody with specificity for antigens on tumor vasculature endothelial cells on one antigen combining site and specificity for the extracellular domains of human TF on the other antigen combining site. The antibody with specificity for human TF was previously shown to bind TF with high affinity without interfering with the factor VIIa complexing event or the TF/VIIa activity (Morrissey et al., 1988).

Instead of using full length human TF, the inventors used a truncated form (tTF), which is devoid of the cytoplasmic as well as the transmembrane domain.

Truncated TF lacks coagulation inducing activity, while still being able to complex factor VIIa, probably because it is not able to complex with a membrane surface upon which the coagulation-initiation complexes, including Factor X, could assemble.

The mouse model used for analyzing the effectiveness of this tumor vasculature endothelial cell specific targeting construct was a recently established model in which MHC class II antigens, that are absent from the vasculature of normal tissues, are expressed on the tumor vasculature through induction by IFN-z that is secreted by the tumor cells (Burrows et al., 1992; Burrows & BR< Thorpe, 1993). It has been demonstrated that anti-class II antibody administered intravenously localizes rapidly and strongly to the tumor vasculature (Burrows et al., 1992) .

The present inventors herein demonstrate that, in a C1300 (Muy) tumor bearing mouse, the anti-MHC Class II/anti-TF bispecific antibody is able to induce coagulation specifically in the vasculature of the tumor when administered together with tTF. Indeed, intravenous administration of the antibody:tTF complex induced rapid thrombosis of tumor vasculature and complete tumor regressions. in 70% of animals. Neither the bispecific antibody alone, nor tTF alone, nor any of the isotype matched control antibodies in the presence or absence of tTF, was able to elicit the same effect. This indicates that the B21-2/10H10 bispecific antibody acts as a "coaguligand" that is capable of bridging target cells and tTF so that tTF can activate factor X and start the coagulation cascade. It also shows the evident success of the coaguligand in treating solid tumors.

G. Pharmaceutical Compositions and Kits Pharmaceutical compositions of the present invention will generally comprise an effective amount of the bispecific coagulating ligand dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated.

Supplementary active ingredients can also be incorporated into the compositions.

1. Parenteral Formulations The bispecific ligands of the present invention will often be formulated for parenteral administration, e.g., formulated for injection via the intravenous, intramuscular, sub-cutaneous or other such routes, including direct instillation into a tumor or disease site. The preparation of an aqueous composition that contains a tumor-targeted coagulant agent as an active ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The bispecific ligands or antibodies can be formulated into a composition in a neutral or salt form.

Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In

many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus ny additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective.

Formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

Suitable pharmaceutical compositions in accordance with the invention will generally include an amount of the bispecific ligand admixed with an acceptable pharmaceutical diluent or excipient, such as a sterile aqueous solution, to give a range of final concentrations, depending on the intended use. The techniques of preparation are generally well known in the art as exemplified by Remington's Pharmaceutical Sciences, 16th Ed. Mack Publishing Company, 1980, incorporated herein by reference. It should be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less that 0.5 ng/mg protein. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

The therapeutically effective doses are readily determinable using an animal model, as shown in the studies detailed herein (see, e.g., Example III).

Experimental animals bearing solid tumors are frequently used to optimize appropriate therapeutic doses prior to translating to a clinical environment. Such models are known to be very reliable in predicting effective anticancer strategies. For example, mice bearing solid tumors, such as used in Example III, are widely used in pre-clinical testing.

The inventors have used mice with C1300 (Mo8) tumors to determine toxicity limits and working ranges of bispecific that give optimal anti-tumor effects with minimal toxicity.

It is currently proposed that effective doses for use in the treatment of cancer will be between about 0.1 mg/kg and about 2 mg/kg, and preferably, of between about 0.8 mg/kg and about 1.2 mg/kg, when administered via the IV route at a frequency of about 1 time per week. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Such optimization and adjustment is routinely carried out in the art and by no means reflects an undue amount of experimentation.

It should be remembered that one aspect of the present invention concerns the delivery of a coagulating agent to a tumor site by administering an uncomplexed bispecific binding ligand that garners an endogenous coagulation factor from the circulation and concentrates it within the tumor site. In these cases, the pharmaceutical compositions employed will contain a ligand having a targeting and coagulant binding region, but will otherwise generally be the same as those described above.

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms are also contemplated, e.g., tablets or other solids for oral administration, time release capsules, liposomal forms and the like. Other pharmaceutical formulations may also be used, dependent on the condition to be treated. For example, topical formulations that are appropriate for treating pathological conditions such as dermatitis and psoriasis; and ophthalmic formulations for diabetic retinopathy.

2. Ingestible Formulations In certain embodiments, active compounds may be administered orally. This is contemplated for agents that are generally resistant, or have been rendered resistant, to proteolysis by digestive enzymes. Such compounds are contemplated to include chemically designed or modified agents; dextrorotatory peptidyl agents; liposomal formulations; and formulations in time release capsules to avoid peptidase and lipase degradation.

For oral administration, the active bispecific compounds may be administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or compressed into tablets, or incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1k of active bispecific coagulant. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60k of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier.

Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustainedrelease preparation and formulations.

3. Liposomal Formulations The bispecific coagulating ligands of the present invention may also be formulated in liposomal preparations if desired. The following information may be utilized in generating liposomal formulations incorporating the present coagulants. Phospholipids form liposomes when dispersed in water, depending on the molar ratio of lipid to water. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state.This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

In addition to temperature, exposure to proteins can alter the permeability of liposomes. Certain soluble proteins such as cytochrome c bind, deform and penetrate the bilayer, thereby causing changes in permeability.

Cholesterol inhibits this penetration of proteins, apparently by packing the phospholipids more tightly. It is contemplated that the most useful liposome formations for use with the present invention will contain cholesterol, or even PEG.

The ability to trap solutes varies between different types of liposomes. For example, multilamellar vesicles (MLVs) are moderately efficient at trapping solutes, but small unilamellar vesicles (suss) are inefficient. SUVs offer the advantage of homogeneity and reproducibility in size distribution, however, and a compromise between size and trapping efficiency is offered by large unilamellar vesicles (L W S). These are prepared by ether evaporation and are three to four times more efficient at solute entrapment than MLVs.

In addition to liposome characteristics, an important determinant in entrapping compounds is the physicochemical properties of the compound itself. Polar compounds are trapped in the aqueous spaces and nonpolar compounds bind to the lipid bilayer of the vesicle.

Polar compounds are released through permeation or when the bilayer is broken, but nonpolar compounds

remain affiliated with the bilayer unless it is disrupted by temperature or exposure to lipoproteins. Both types show maximum efflux rates at the phase transition temperature.

Liposomes interact with cells via four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents. It often is difficult to determine which mechanism is operative and more than one may operate at the same time.

The fate and disposition of intravenously injected liposomes depend on their physical properties, such as size, fluidity and surface charge. They may persist in tissues for hours or days, depending on their composition, and half lives in the blood range from minutes to several hours. Larger liposomes, such as MLVs and Lugs, are taken up rapidly by phagocytic cells of the reticuloendothelial system, but physiology of the circulatory system restrains the exit of such large species at most sites. They can exit only in places where large openings or pores exist in the capillary endothelium, such as the sinusoids of the liver or spleen. Thus, these organs are the predominate site of uptake. On the other hand, SUVs show a broader tissue distribution but still are sequestered highly in the liver and spleen.In general, this in vivo behavior dictates that liposomes concentrate only in those organs and tissues accessible to their large size. As this clearly includes the blood, this is not a limitation to their combined use with the present invention.

In other embodiments, the bispecific components of the invention may be admixed with the liposome surface to direct the drug contents to the specific antigenic receptors located on the target cell surface.

Carbohydrate determinants (glycoprotein or glycolipid cell-surface components that play a role in cell-cell recognition, interaction and adhesion) may also be used as recognition sites as they have potential in directing liposomes to particular cell types. Mostly, it is contemplated that intravenous injection of liposomal preparations would be used, but other routes of administration are also conceivable.

4. Topical Formulations The formulation of bispecific coagulants for topical use, such as in creams, ointments and gels is also contemplated. The preparation of oleaginous or watersoluble ointment bases is also well known to those in the art. For example, these compositions may include vegetable oils, animal fats, and more preferably, semisolid hydrocarbons obtained from petroleum.

Particular components used may include white ointment, yellow ointment, cetyl esters wax, oleic acid, olive oil, paraffin, petrolatum, white petrolatum, spermaceti, starch glycerite, white wax, yellow wax, lanolin, anhydrous lanolin and glyceryl monostearate.

Various water-soluble ointment bases may also be used, including glycol ethers and derivatives, polyethylene glycols, polyoxyl 40 stearate and polysorbates. Even delivery through the skin may be employed if desired, e.g., by using transdermal patches, iontophoresis or electrotransport.

5. Ophthalmic Formulations The bispecific coagulating ligands of the present invention may also be formulated into pharmaceutical compositions suitable for use as ophthalmic solutions.

Such ophthalmic solutions are of interest, for example, in the treatment of diabetic retinopathy. Thus, for the treatment of diabetic retinopathy a bispecific conjugate of this invention would be administered to the eye of the subject in need of treatment in the form of an ophthalmic preparation prepared in accordance with conventional pharmaceutical practice, see for example "Remington's Pharmaceutical Sciences" 15th Edition, pages 1488 to 1501 (Mack Publishing Co., Easton, PA).

The ophthalmic preparation will contain a novel bispecific coagulant or a pharmaceutically acceptable salt thereof in a concentration from about 0.01 to about 1% by weight, preferably from about 0.05 to about 0.5k in a pharmaceutically acceptable solution, suspension or ointment. Some variation in concentration will necessarily occur, depending on the particular compound employed, the condition of the subject to be treated and the like, and the person responsible for treatment will determine the most suitable concentration for the individual subject. The ophthalmic preparation will preferably be in the form of a sterile aqueous solution containing, if desired, additional ingredients, for example preservatives, buffers, tonicity agents,

antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents and the like.

Suitable preservatives for use in such a solution include benzalkonium chloride, benzethonium chloride, chlorobutanol, thimerosal and the like. Suitable buffers include boric acid, sodium and potassium bicarbonate, sodium and potassium borates, sodium and potassium carbonate, sodium acetate, sodium biphosphate and the like, in amounts sufficient to maintain the pH at between about pH 6 and pH 8, and preferably, between about pH 7 and pH 7.5. Suitable tonicity agents are dextran 40, dextran 70, dextrose, glycerin, potassium chloride, propylene glycol, sodium chloride, and the like, such that the sodium chloride equivalent of the ophthalmic solution is in the range 0.9 plus or minus 0.2%.

Suitable antioxidants and stabilizers include sodium bisulfite, sodium metabisulfite, sodium thiosulfite, thiourea and the like. Suitable wetting and clarifying agents include polysorbate 80, polysorbate 20, poloxamer 282 and tyloxapol. Suitable viscosity-increasing agents include dextran 40, dextran 70, gelatin, glycerin, hydroxyethylcellulose, hydroxmethylpropylcellulose, lanolin, methylcellulose, petrolatum, polyethylene glycol, polyvinyl alcohol, polyvinylpyrrolidone, carboxymethylcellulose and the like. The ophthalmic preparation will be administered topically to the eye of the subject in need of treatment by conventional methods, for example in the form of drops or by bathing the eye in the ophthalmic solution.

6. Therapeutic Kits The present invention also provides therapeutic kits comprising the bispecific coagulating ligands described herein. Such kits will generally contain, in suitable container means, a pharmaceutically acceptable formulation of at least one bispecific ligand in accordance with the invention. The kits may also contain other pharmaceutically acceptable formulations, such as those containing additional bispecific coagulating ligands, generally those having a distinct targeting component; extra uncomplexed coagulation factors; bispecific antibodies, T cells, or other functional components for use in, e.g., antigen induction; components for use in antigen suppression, such as a cyclosporin; distinct anti-tumor site antibodies or immuntoxins; and any one or more of a range of chemotherapeutic drugs.

Preferred agents for use in combination kits are inducing agents capable of inducing disease-associated vascular endothelial cells to express a targetable antigen, such as E-selectin or an MHC Class II antigen.

Inducing agents can include T cell clones that bind disease or tumor antigens and that produce IFN-y.

Preferred inducing agents include bispecific antibodies that bind to disease or tumor cell antigens and to effector cells capable of inducing target antigen expression through the elaboration of cytokines.

As such, the present invention further includes kits that comprise, in suitable container means, a first pharmaceutical composition comprising a bispecific antibody that binds to an activating antigen on an effector cell surface, i.e., a monocyte/ macrophage, mast cell, T cell or NK cell, and to an antigen on the cell surface of disease cell; and a second pharmaceutical composition comprising a bispecific ligand that comprises a first binding region that binds to an endothelial cell antigen induced by an activated effector cell, or cytokine therefrom, where the first binding region is operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor.

Kits including a first pharmaceutical composition that comprises a bispecific antibody that binds to the activating antigen CD14, CD16 (FcR for IgE), CD2, CD3, CD28 or the T-cell receptor antigen are preferred, with CD14 or CD28 binding bispecific antibodies being more preferred. Activation of monocyte/macrophages or mast cells via CD14 or CD16 binding results in IL-1 production that induces E-selectin; whereas activation of T cells via CD2, CD3 or CD28 binding results in IFN- production that induces MHC class II. Kits that include a second pharmaceutical composition that comprises a bispecific ligand that comprises a first binding region that binds to E-selectin or to an MHC Class II antigen are therefore also preferred.

The kits may have a single container means that contains the bispecific coagulating ligand, with or without any additional components, or they may have distinct container means for each desired agent. Kits comprising the separate components necessary to make a bispecific coagulating ligand are also contemplated.

When the components of the kit are provided in one or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. However, the components of the kit may be provided as dried powder(s). When reagents or components are provided as a dry powder,

the powder can be reconstituted by the addition of a suitable solvent.

It is envisioned that the solvent may also be provided in another container means.

The container means of the kit will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the bispecific coagulating ligand, and any other desired agent, may be placed and, preferably, suitably aliquoted.

Where additional components are included, the kit will also generally contain a second vial or other container into which these are placed, enabling the administration of separated designed doses. The kits may also comprise a second/third container means for containing a sterile, pharmaceutically acceptable buffer or other diluent.

The kits may also contain a means by which to administer the bispecific ligand to an animal or patient, e.g., one or more needles or syringes, or even an eye dropper, pipette, or other such like apparatus, from which the formulation may be injected into the animal or applied to a diseased area of the body. The kits of the present invention will also typically include a means for containing the vials, or such like, and other component, in close confinement for commercial sale, such as, e.g., injection or blow-molded plastic containers into which the desired vials and other apparatus are placed and retained.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I SYNTHESIS OF A BISPECIFIC COAGULATING ANTIBODY The present example describes the synthesis of a bispecific antibody capable of specifically directing a coagulant to a tumor site, i.e., a "coaguligand".

A. Materials and Methods 1. Reagents Pepsin (A; EC 3.4.23.1), Ellmans reagent (ER; 5,5' -dithio-bis (2-nitrobenzoic acid, DNTB), 2-mercaptoethanol (2-ME), sodium arsenite (NaAsO2) and rabbit brain thromboplastin (acetone powder) were obtained from Sigma Chemical Co., St. Louis MO. Sephadex G-25 and G-100 were obtained from Pharmacia LKB (Piscataway,NJ).

2. Human Truncated Tissue Factor (tTF) Recombinant human truncated TF (tTF) was prepared by one of two different methods.

Method I: Construction of the E. coli Expression Vector.

The cDNA coding for tTF (residues 1-218) was amplified by PCR using the primers 5' -GAAGAAGGGATCCTGGTGCCTCGTGGTTCTGGCACTACAAATACT-3' (5'-primer; SEQ ID NO:28) and 5' -CTGGCCTCAAGCTTAACGGAATTCACCTTT-3' (3'-primer; SEQ ID NO:29) which allowed the addition of the coding sequence for a thrombin cleavage site upstream of the cDNA. The PCR products were cleaved using BamHI and HindIII and ligated between the BamHI and HindIII sites of the expression vector pTrcHisC (Invitrogen).

DH5a cells were transformed with the ligation mixture and recombinant plasmids were isolated after selection in the presence of ampicillin. The E. coli strain BL21 was transformed with the recombinant plasmid pTrcHisC-tTF and the resultant transformants were used for protein expression.

Method I: Expression, Refolding and Purification of tTF from E. coli. The poly(his)-tTF fusion protein was expressed using BL21 cells transformed with pTrc-HisCtTF. Inoculant cultures (10 ml in LB medium) were grown overnight shaking at 370C.

Inoculant cultures were added to growth medium which were then grown shaking at 370C. When the optical density at 550 nm had reach ca. 0.5, 10 ml of 100 mM isopropyl-P-D-thiogalactopyranoside was added. Shaking was continued at 370C for ca. 20 h (to stationary phase).

The cells were harvested by centrifugation (10,000 x g, 20 min.) and the inclusion bodies were isolated as follows (quantities of reagents are per gram of cell paste). The cell paste was suspended in 4 ml of 10 mM Tris, pH 7.5, 150 mM NaC1, 1 mM MgCl2, 0.17 mg/ml PMSF, 2 mg/ml hen egg white lysozyme (Sigma). Benzonase (250 units, EM Science) was added the suspension was mixed gently at room temperature for 1.5 h then centrifuged at 12,000 g for 15 min.

The pellet was resuspended in 10 mM Tris, pH 7.5, 1 mM EDTA, 3% NP40 (2 ml), sonicated for 1 min at 50% power and centrifuged at 12,000 x g for 20 min. The pellet was resuspended in water, sonicated for 20-30 seconds at 50% power and centrifuged at 12,000 x g for 20 min. The water wash was repeated and the final pellet, highly enriched for the inclusion bodies, was suspended in 6 M guanidinium chloride, 0.5 M NaCl, 20 mM phosphate, 10 mM P-mercaptoethanol, pH 8.0 (9 ml per gram of inclusion bodies) by gentle mixing at room temperature overnight.

The suspension was centrifuged at 12,000 x g for 20 min and the supernatant was loaded onto a nickel nitriloacetic acid (Ni-NTA, Qiagen) column. The column was washed successively with the same 6 M guanidinium chloride buffer at pH 8 then pH 7, then the protein was eluted by decreasing the pH to 4.

Ni-NTA column fractions containing the fusion protein were combined and dithiothreitol was added to 50 mM. The solution was held at room temperature overnight then diluted to a protein concentration of ca. 1 mg/ml in 6 M urea, 50 mM Tris, 0.02W sodium azide, pH 8.0 and dialyzed at 40C overnight against 10-20 volumes of the same buffer. The buffer was changed to 2 M urea, 50 mM Tris, 300 mM NaCl, 2.5 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.02% sodium azide, pH 8.0 (folding buffer) - Dialysis was continued for 2 more days, the buffer was replaced by fresh folding buffer and dialysis was continued for 2 more days.

The solution was then dialyzed extensively against 20 mM TEA (pH 7.5), removed from the dialysis bag, treated with human thrombin (ca. 1 part per 500 parts recombinant protein w/w) overnight at room temperature, and loaded onto a HR-10/10 mono-Q anion exchange column.

tTF protein was eluted using a 20 mM TEA buffer containing NACl in a concentration increasing linearly from 0 to 150 mM over 30 minutes (flow rate 3 ml/min).

Method II: Preparation of tTF complimentary DNA (cDNA).

RNA from J-82 cells (human bladder carcinoma) was used for the cloning of tTF. Total RNA was isolated using the GlassMaxTM RNA microisolation reagent (Gibco BRL). The RNA was reverse transcribed to cDNA using the GeneAmp RNA PCR kit (Perkin Elmer). tTF cDNA was amplified using the same kit with the following two primers: 5' primer: 5' GTC ATG CCA TGG CCC TGG TGC CTC GTG CTT CTG GCA CTA CAA ATA CT 3' primer: 5' TGA CAA GCT TAT TCT CTG AAT TCC CCC TTT CT (SEQ ID NO:2) The underlined sequences codes for the N-terminus and C-terminus of tTF. The rest of the sequence in the 5' primer is the restriction site for Ncol allowing the cloning of tTF into the expression vector and codes for a cleavage site for thrombin. The sequence in the 3' primer is the HindIII site for cloning tTF into the expression vector. PCR amplification was performed as suggested by the manufacturer.Briefly, 75 yM dNTP; 0.6 yM primer, 1.5 mM MgC12 were used and 30 cycles of 30" at 950C, 30" at 550C and 30" at 720C were performed.

Method II. Vector Constructs. E. coli expression vector H6 pQE-60 was used for expressing tTF (Lee et. al., 1994). The PCR amplified tTF cDNA was inserted between the Ncol and HindIII site. H6 pQE-60 has a built-in (His)6 encoding sequence such that the expressed protein has the sequence of (His)6 at the N-terminus, which can be purified on the Ni-NTA column.

Method II. tTF Purification. tTF containing H6 pQE-60 DNA was transformed to E. coli TG-1 cells. The cells were grown to OD600 = 0.5 and IPTG was added to 30 4M to induce the tTF production. The cells were harvested after shaking for 18 h at 300C. The cell pellet was denatured in 6 M Gu-HCl and the lysate was loaded onto a Ni-NTA column (Qiagen). The bound tTF was washed with 6 M urea and tTF was refolded with a gradient of 6 M - 1 M urea at room temperature for 16 h. The column was washed with wash buffer (0.05 Na H2 PO4, 0.3 M NaCl, 10% glycerol) and tTF was eluted with 0.2 M Imidozole in wash buffer.

The eluted tTF was concentrated and loaded onto a G75 column. tTF monomers were collected and treated with thrombin to remove the H6 peptide. This was done by adding 1 part of thrombin (Sigma) to 500 parts of

tTF (w/w), and the cleavage was carried out at room temperature for 18 h. Thrombin was removed from tTF by passage of the mixture through a Benzamidine Sepharose 6B thrombin affinity column (Pharmacia).

The tTF had identical ability to recombinant tTF from yeast or Chinese hamster ovary cells to bind factor VIIa and to enhance the catalytic activity of VIIa (Ruf et al., 1991). When analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, it ran as a single component having a molecular weight of approximately 24 kD.

3. Monoclonal Antibodies B21-2 (TIB-229) hybridoma and SFR8-B6 hybridoma (HB-152, hereafter referred to as SFR8) were obtained from the ATCC. Both hybridomas secreted rat IgG2b antibodies, which were purified from culture supernatant by protein G affinity chromatography. The B21-2 antibody reacts with I-Ad antigen expressed on A20 cells as well as on the vasculature of the C1300 (Muy) transfectant tumors grown in BALB/c/nu/nu mice. SFR8 antibody is directed against the HLA-Bw6 epitope and serves as an isotype matched negative control for the B21-2 antibody.

TF9/10H10 (referred to as 10H10), a mouse IgG1, is reactive with human TF without interference of TF/factor VIIa activity and was produced as described by Morrissey et al. (1988).

The cell line MRC OX7 (referred to as OX7) was obtained from Dr. A. F. Williams (MRC Cellular Immunology Unit, University of Oxford, Oxford, England). It secretes the OX7 antibody, a mouse IgG1 antibody that recognizes the Thy 1.1 antigen on T lymphocytes. It was used as an isotype matched negative control for TF9/10H10.

All antibodies were purified from culture supernatant by protein G affinity chromatography.

4. Synthesis of BisPecific Antibodies F(ab')2 fragments were obtained by digesting their respective IgGs with 2% (w/v) pepsin for 5-9 hrs at 37 OC and purification of the fragments by Sephadex G100 chromatography. Synthesis of the bispecific antibodies B21-2/IOH10, SFR8/IOH10 and B21-2/OX7 was carried out according to the method of Brennan et al. (1985) with minor modifications.

The bispecific antibodies B21-2/10H10, SFR8/IOH10, OX7/10H10 and B21-2/OX7 were synthesized according to the method of Brennan et al. (1985) with minor modifications.

In brief, F(ab')2 fragments were obtained from the IgG antibodies by digestion with pepsin and were purified to homogeneity by chromatography Sephadex G100. F(ab')2 fragments were reduced for 16 h at 200C with 5mM 2mercaptoethanol in 0.1 M sodium phosphate buffer, pH 6.8, containing 1 mM EDTA (PBSE buffer) and 9 mM NaAs02.

Ellman's reagent (ER) was added to give a final concentration of 25 mM and, after 3 h at 200C, the Ellman's-derivatized Fab' fragments (Fab'-ER) were separated from unreacted ER on columns of Sephadex G25 in PBSE.

To form the bispecific antibody, Fab'-ER derived from one antibody was concentrated to approximately 2.5 mg/ml in an ultrafiltration cell and was reduced with 10 mM 2-mercaptoethanol for 1 h at 200C. The resulting Fab'-SH was filtered through a column of Sephadex G25 in PBSE and was mixed with equal molar quantities of Fab'-ER prepared from the second antibody. The mixtures were concentrated by ultrafiltration to approximately 3 mg/ml and were stirred for 16 h at 200C. The products of the reaction were fractionated on columns of Sephadex G100 and the fractions containing the bispecific antibody (110 kDa) were concentrated to 1 mg/ml, and were stored at 40C in 0.02% sodium azide.

B. Results 1. Analysis of Bispecific Antibodies The molecular weight of the F(ab')2 fragments and bispecific preparations were determined by SDS-Page electrophoresis with 4-15W gradient gels using the Pharmacia LKB-Phastsystem (Pharmacia LKB, Piscataway, NJ). Bispecificity as well as the percentage of heterodimer vs homodimer was determined by FACS analysis (Example II).

Analysis of the bispecific antibodies by SDS-Page electrophoresis (and by FACS, Example II) demonstrated that the B21-2/10H10 bispecific contained less than 4% homodimer of either origin and < 10% fragments with a molecular weight of 140 kD or 55 kD. Approximately 10% of the preparation consisted of 140 kD fragments, probably being a F(ab')2 construct with an extra light chain (of either origin) attached.

EXAMPLE II COAGULATING ANTIBODY BINDING AND FUNCTION IN VITRO The present example shows the bispecificity of the coagulating antibody (coaguligand) and demonstrates that specific binding, cellular delivery and coagulation is achieved in vitro using the coaguligand.

A. Materials and Methods 1. Cells The A20 cell line, which is an I-Ad positive BALB/c B-cell lymphoma, was purchased from the American Type Culture Collection (ATCC; Rockville, MD; TIB-208). A20 cells were grown in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), 0.2 mM L-glutamine, 200 units/ml penicillin and 100 yg/ml streptomycin, 18 mM Hepes, 0.1 mM non-essential amino acids mix and lmM sodium pyruvate (medium hereafter referred to as complete DMEM; all reagents obtained from Life Technologies, Gaitherburg, MD). 2-ME is added to complete DMEM to a final concentration of 0.064 mM for A20 cells. Cultures were maintained at 370C in a humidified atmosphere of 90% air/10% CO2.

J82, a human gall bladder carcinoma expressing TF, was obtained from the ATCC (HTB-1). The cells grew adherently in complete DMEM.

The C1300 neuroblastoma cell line was established from a spontaneous tumor, which arose in an A/Jax mouse (Dunham & Stewart, 1953). The C1300 (mum) 12 line, hereafter referred to as C1300 (Muy) was derived by transfection of C1300 neuroblastoma cells with the murine IFN-- gene using the IFN- expression retrovirus pSVX (Muy bAs) (Watanabe et al., 1989). The IFN- expression retrovirus was obtained from Dr. Y. Watanabe (Department of Molecular Microbiology, Kyoto University, Japan).

C1300(Mur)12 cells were maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 2.4 mM L-glutamine, 200 units/ml penicillin, 100 yg/ml streptomycin, 100 M nonessential amino acids, 1 M sodium pyruvate, 18yM HEPES and 1 mg/ml G418 (Geneticin; Sigma). Cultures were maintained at 370C in a humidified atmosphere of 90% air/10% CO2.

The Thy 1.1-expressing AKR-A mouse T lymphoma cell line was obtained from Prof. Dr. I. MacLennan (Department of Experimental Pathology, Birmingham University, Birmingham, England) and were grown in complete DMEM.

2. Indirect immunofluorescence A20 cells were resuspended in PBS/0.2% BSA/0.02% Na-azide (hereafter referred to as FACS buffer) at 4 x 106 cells/ml. J82 cells were released from the flask under mild conditions using PBS/EDTA (0.2 k w/v) and resuspended at 4 x 106 cells/ml in FACS buffer. 50 il of cell suspension was added to 50 yl of optimal serial dilutions of the primary antibody in wells of a round-bottomed 96 well plate. After incubation at RT for 15 min, the cells were washed with FACS buffer 3 times.

After removing the final supernatant, 50 I of the secondary antibody conjugated to fluorescein isothiocyanate (FITC), in a 1 in 20 dilution in FACS buffer, was added to the cells. The cells were incubated for a further 15 min at RT and washed 3 times with FACS buffer. Cell associated fluorescence was measured on a FACScan (Becton Dickenson, Fullerton, CA). Data were analyzed using the Lysis II program. When FITC-anti-rat immunoglobulin was used as the secondary antibody, normal mouse serum (10% v/v) was added to block non-specific cross reactivity with the mouse cells.

3. Radiolabeling of Proteins Proteins were labeled with 125Iodine according to the chloramine T protocol described by Mason & Williams (1980), (protocol 2). The iodinated product was purified on G25 and stored at - 70 C in the presence of;5% DMSO and 5 mg/ml bovine IgG in the case of the monoclonal fragments and 5% DMSO and 5 mg/ml BSA in the case of tTF.

Specific activity ranged between 2.5 yCi/yg and 4.8 yCi/yg.

4. Binding studies Human tTF was labelled with 125I to a specific activity of 2.5-4.8 Ci/ g using the chloramitie T procedure (Protocol 2) described by Mason and Williams (1980). A suspension of A20 cells at 2 x 106 cells/ml in PBS containing 2 mg/ml BSA and b.02 sodium azide was distributed in 50 I volumes into the wells of 96 well round-bottomed microtiter plates. To the wells were added 25 yl of bispecific antibodies prepared over a range of concentrations (8 to 0.02 yg/ml) in the same buffer.

25 I of 125I-tTF at 8 yg/ml in the same buffer were added to each well, giving a molar excess of tTF. The plates were shaken and incubated for 1 hr at 40C. The cells were then washed 3x in the plates with 0.9k (w/v) NaCl containing 2 mg/ml BSA. The contents of the wells were pipetted over a 10:11 (v/v) mixture of dibutyl phthalate and bis(2-ethylhexyl)phthalate oils in microcentrifuge tubes. The tubes were centrifuged for

1.5 min at 7500 g and were snap frozen in liquid nitrogen. The tips containing the cells were cut off.

The radioactivity in the cell pellet and in the supernatant was measured in a gamma counter.

5. Coagulation Assav An identical microplate to that used for the binding assay above was set up on the same occasion, except that non-labelled tTF was added instead of 125I-tTF. After the Ih incubation at 40C, the cells were washed 3x as before and were resuspended in 75 yl of 0.9k NaCl containing 2 mg/ml BSA and 12.5 mM CaCl2. The contents of the wells were transferred to 5 ml clear plastic tubes and were warmed to 370C. To each tube was added 30 y1 of citrated mouse plasma at 370C. The time for the first fibrin strands to form was recorded.

B. Results 1. Antibody Bisecificitv For SFR8/10H10 bispecificity was shown by FACS using J82 cells (TF positive) as target cells and FITC-anti-mouse immunoglobulin to demonstrate 10H10 presence. FITC-anti-rat immunoglobulin was used to demonstrate the presence of SFR8. The mean fluorescence intensity-versus-concentration curves were coincident for both stains, demonstrating that both the mouse and the rat arm are present in the bispecific preparation.

2. Antibody Binding Binding studies with 125Iodine labeled B21-2 Fab' and SFR8 Fab' showed that the concentration at which saturation of binding of B21-2 Fab' to A20 cells is reached is 21.5 nM. The SFR8 Fab' bound non-specifically to A20 cells, with the number of molecules bound per cell being less than 50,000 at 21.5 nM versus 530,000 for B21-2 Fab'.

3. Coagulant Deliverv and Tethering To study the capability of bridging tTF to A20 cells through the B21-2/10H10 bispecific antibody as compared to the control bispecific antibodies, A20 cells were incubated with bispecific antibody and a 125I-tTF concentration range as indicated. Saturation was attained at concentrations of bispecific antibody of 10 nM (1 yg/ml) or more, when an average of 310,000 molecules of tTF were bound to each A20 cell. The binding was specific since no tTF binding was mediated by either of the isotype-matched control bispecific antibodies, SFR8/10H10 or B21-2/OX7, which had only one of the two specificities needed for tethering tTF (FIG. 1) .

4. Coagulation To investigate whether tTF bound to A20 cells through a bispecific antibody was able to induce coagulation, the inventors first incubated A20 cells with 21.5 nM bispecific antibody and 69 nM tTF. The resulting effect on the coagulation time is shown in Table VII.

These first studies showed that A20 cells coated with a complex of B21-2/10H10 and tTF were capable of inducing fibrin formation: it shortened coagulation time from 140 sec (the time for mouse plasma in CaC12 to coagulate in the absence of added antibodies or TF under the specific conditions used) to 60 sec. In contrast, the control bispecific antibodies did not induce activation of coagulation: in these cases coagulation time was 140 sec.

Later studies confirmed and extended the initial results. Mouse plasma added to A20 cells to which tTF had been tethered with B21-2/10H10 coagulated rapidly.

Fibrin strands were visible 36 seconds after adding the plasma as compared with 164 seconds in plasma added to untreated A20 cells (Table VII). Only when tTF had been tethered to the cells was coagulation induced: no effect on coagulation time was seen with cells incubated with of tTF alone, homodimeric F(ab')2 Fab' fragments or bispecific antibodies having only one of the two specificities needed for tethering tTF.

A linear relationship existed between the logarithm of the average number of tTF molecules tethered to each A20 cell and the rapidity with which those cells induced coagulation of mouse plasma (FIG. 2). Cells bearing 300,000 molecules of tTF per cell induced coagulation in 40 secs but even with 20,000 molecules per cell coagulation was significantly faster (140 secs) than it was with untreated cells (190 secs).

Table VII. Coagulation of mouse plasma induced by tethering tTF to A20 cells with bispecific antibody.

Reagents added Coagulation time2 (sec) None 164 + 4 B21-2/10H10 + tTF 36 + 2 B21-2/10H10 163 + 2 tTF 163 i 3 B21-2/OX7 + tTF 165 + 4 SFR8/10H10 + tTF 154 + 5 10H10 F(ab')2 + tTF 160 + 3 10H10 Fab' + tTF 162 i 2 B21-2 F(ab')2 + tTF 168 + 4 B21-2 Fab' + tTF 165 i 4 1Bispecific antibodies F(ab')2 and Fab' fragments (0.33 g/105 cells/100 y1) and tTF (0.17 ijg/lO5cells/100 y1) were incubated with A20 cells for 1h at 40C in 0.2k w/v sodium azide. The cells were washed, warmed to 370C, calcium and plasma were added

and the time for the first fibrin strands to form was recorded.

2Arithmetic mean of triplicate determinations + standard deviation EXAMPLE III SPECIFIC TUMOR VASCULATURE SPECIFIC COAGULATION IN VIVO The present example describes the specific coagulation of tumor vasculature in vivo that results following the administration of the bispecific antibody coaguligand as a delivery vehicle for human tissue factor.

A. Materials and Methods 1. Reagents Mouse blood was obtained by heartpuncture and collected in 1/10 volume of 3 . 8k buffered citrate. The blood was centrifuged for 10 min at 3000g and the plasma snap frozen in small aliquots and stored at - 70 OC.

2. Animals BALB/c nu/nu mice were obtained from Simonsen (Gilroy, CA) and maintained under SPF conditions.

3. C1300 (mum) Mouse Model and Treatment The tumor model was as previously described (Burrows et al., 1992; Burrows & Thorpe, 1993) with three refinements. First, a different antibody, B21-2, was used. This antibody recognizes I-Ad but not I-Ed, unlike the previously used M5/114 antibody which recognizes both molecules. The B21-2 antibody has.an approximately 10fold better affinity than M5/114. Second, a subline of the previously used C1300(Muy)12 line was used which grew continuously in BALB/c nu/nu mice. The C1300 (Mu) 12 cells used previously had to be mixed with untransfected C1300 cells in order to form continuously growing tumors.

The new subline, designated C1300(Muy) tIP3, will be referred to hereafter as C1300(Mu). Third, it was unnecessary to add tetracycline to the mice's drinking water to prevent gut bacteria from inducing I-Ad on the gastrointestinal epithelium. Unlike immunotoxins, coaguligands do not damage I-Ad-expressing intestinal epithelium.

For establishment of solid tumors, 1.5 x 107 C1300 (mum) cells were injected subcutaneously into the right anterior flank of BALB/c nu/nu mice. When the tumors had grown to 0.8 cm in diameter, mice were randomly assigned to different treatment groups each containing 7-8 mice.

Coaguligands were prepared by mixing bispecific antibodies (140 yg) and tTF (110 yg) in a total volume of 2.5 ml of 0.9k NaCl and leaving them at 40C for one hour.

Mice then received intravenous injections of 0.25 ml of this mixture (i.e. 14 yg of bispecific antibody plus 11 yg of tTF). Other mice received 14 yg of bispecific antibodies or 11 g of tTF alone. The injections were performed slowly into one of the tail veins over approximately 45 sec and were followed with a second injection of 200 yl of saline into the same vein. This injection procedure was adopted to prevent thrombosis of the tail vein which was seen if mice were rapidly injected (1-2 sec). Seven days later, the treatments were repeated.

Perpendicular tumor diameters were measured at regular intervals and tumor volumes were estimated according to the following equation: volume = smaller diameter2 x larger diameter x w/6 Differences in tumor volume were tested for statistical significance using the Mann-Whitney-Wilcoxon nonparametric test for two independent samples (Gibbons, 1976) .

For histopathological analyses, animals were anesthetized with metophane at various times after treatment and were exsanguinated by perfusion with heparinized saline. 500 IU of heparin were i.v.

injected, the animal anesthetized with metofane and the systemic circulation perfused with PBS at a flow rate of 0.6 mls/min until the liver had been cleared of blood.

The tumor and normal tissues were excised and formal in fixed (4k v/v). Paraffin sections of the tissues were cut and stained with the standard Martius Scarlet Blue (MSB) trichrome technique for detection of fibrin, and with hematoxylin and eosin stain for cell morphology.

B. Results 1. Improved Tumor Model To improve the C1300 (mum) tumor model as described before (Burrows et al., 1992), the inventors subcloned the C1300 (Muy) cell line into a cell line that can grow without being mixed with its parental cell, C1300, but still express the I-Ad MHC Class II antigen on the endothelial cells of the tumor. The inventors used an anti-I-Ad antibody (B21-2) that has a 5-10 fold higher

affinity for its antigen than the initial anti-I-Ad antibody (M5/114.15.2) used in this model as determined by FACS. In vivo distribution studies with this new anti-I-Ad antibody showed the same tissue distribution pattern as did M5/114.15.2. Intense staining with B21-2 was seen in tumor vascular endothelium, light to moderate staining in Kuppfer cells in the liver, the marginal zones in the spleen and some areas in the small and large intestines.Vessels in other normal tissues were unstained.

2. Determination of Suitable In vivo Doses The maximum tolerated dose was 16 yg B21-2/10H10 plus 11 yg tTF injected intravenously into the tail vein of mice. At this dose, mice lost no weight and had normal appearance and activity levels. At a higher dose of 20 yg B21-2/10H10 plus 16 yg tTF, two of ten mice developed localized dermal hemorrhages which eventually resolved. The lower dose was adopted for in vivo studies. Truncated TF itself was not toxic at 50 yg, given intravenously.

3. Specific Coagulation and Infarction in Tumor Vasculature Intravenous administration of a coaguligand composed of B21-2/10H10 (20 yg) and tTF (16 yg) to mice bearing solid C1300 (Muy) tumors caused tumors to assume a blackened, bruised appearance within 30 minutes. A histological study of the time course of events within the tumor revealed that 30 minutes after injection of coaguligand all vessels in all regions of the tumor were thrombosed (FIG. 3B). Vessels contained platelet aggregates, packed red cells and fibrin. At this time, tumor-cells were healthy, being indistinguishable morphologically from tumor cells in untreated mice (FIG.

3A).

By 4 hours, signs of tumor cell distress were evident. The majority of tumor cells had begun to separate from one another and had developed pyknotic nuclei (FIG. 3C). Erythrocytes were commonly observed in the tumor interstitium. By 24 hours, advanced tumor necrosis was visible throughout the tumor (FIG. 3D). By 72 hours, the entire central region of the tumor had compacted into morphologically indistinct debris.

In one of three of the tumors examined, a viable rim of tumor cells 5-10 cell layers thick was visible on the outskirts of the tumor where it was infiltrating into surrounding normal tissues. Immunohistochemical examination of serial sections of the same tumor revealed that the vessels in the regions of tumor infiltration lacked class II antigens.

Tumors from control mice which had received B212/10H10 bispecific antibody (20 yg) alone 30 minutes or 24 hours earlier showed no signs of infarction. Tumors from mice which received tTF (16 yg), alone or in combination with B21-2/OX7 or SFR8/IOH10, showed no signs of infarction 30 min after injection but 24 hours after injection, occasional vessels (about 20% of vessels overall) in the tumor were infarcted. These appeared to be most prevalent in the core of the tumor.

No thrombi or morphological abnormalities were visible in paraffin sections of liver, kidney, lung, intestine, heart, brain, adrenals, pancreas and spleen taken from tumor-bearing mice 30 minutes, 4 hours and 24 hours after. administration of coaguligand or tTF.

4. Tumor Recrressions of Solid Tumors FIG. 4 shows the results of a representative antitumor study in which a coaguligand composed of B212/10H10 and tTF was administered to mice with 0.8 cm diameter tumors. The tumors regressed to approximately half their pretreatment size. Repeating the treatment on the 7th day caused the tumors to regress further, usually completely. In 5/7 animals, complete regressions were obtained. Two of the mice subsequently relapsed four and six months later. These anti-tumor effects are statistically highly significant ($P < 0.001$) when compared with all other groups.

Tumors in mice treated with tTF alone or with tTF mixed with the isotype-matched control bispecific antibodies, SFR8/10H10 or B21-2/OX7, grew more slowly than those in groups receiving antibodies or diluent alone. These differences were statistically significant ($P < 0.05$) on days 12-14. Thus, part of the anti-tumor effects seen with the B21-2/10H10 coaguligand are attributable to a slight non-specific action of tTF itself.

At the end of the study, two mice which had been treated with diluent alone and which had very large tumors of 2.0 cm3 and 2.7 cm3 (i.e. 10-15 of their body weight) were given coaguligand therapy. Both had complete remissions although their tumors later regrew at the original site of tumor growth.

C. Discussion The present studies show that soluble human tTF, possessing practically no ability to induce

coagulation, became a powerful thrombogen for tumor vasculature when targeted by means of a bispecific antibody to tumor endothelial cells. In vitro coagulation studies showed that the restoration of thrombotic activity of tTF is mediated through its cross-linking to antigens on the cell surface.

tTF binds factors VII and VIIa with high affinity and enhances the catalytic activity of VIIa but does not induce coagulation of plasma because the tTF:VIIa complex has to be associated with a membrane surface for efficient activation of factors IX and X (Ruf et al., 1991; Krishnaswamy et al., 1992). Tethering of tTF:VIIa to the cell surface by means of a bispecific antibody restores its ability to induce coagulation by bringing the tTF:VIIa into close proximity to the membrane: the membrane phospholipid provides the surface on which the coagulation-initiation complexes with factors IX or X can assemble and efficiently produce intermediates in the clotting process.

Administration of a coaguligand directed against class II to mice having tumors with class II-expressing vasculature caused rapid thrombosis of blood vessels throughout the tumor. This was followed by infarction of the tumor and complete tumor regressions in a majority of animals. In those animals where complete regressions were not obtained, the tumors grew back from a surviving rim of tumor cells on the periphery of the tumor where it had infiltrated into the surrounding normal tissues. The vessels at the growing edge of the tumor lacked class II antigens, thus explaining the lack of thrombosis of these vessels by the coaguligand. It is likely that these surviving cells would have been killed by coadministering a drug acting on the tumor cells themselves, as was found previously (Burrows & Thorpe, 1993).

The anti-tumor effects of the coaguligand were similar in magnitude to those obtained in the same tumor model with an immunotoxin composed of anti-class II antibody and deglycosylated ricin A-chain (Burrows & BR< Thorpe, 1993). One difference between the two agents is their rapidity of action. The coaguligand induced thrombosis of tumor vessels in less than 30 minutes whereas the immunotoxin took 6 hours to achieve the same effect. The immunotoxin acts more slowly because thrombosis is secondary to endothelial cell damage caused by the shutting down of protein syntheses.

A second and important difference between the immunotoxin and the coaguligand is that they have different toxic side effects. The immunotoxin caused a lethal destruction of class II-expressing gastrointestinal epithelium unless antibiotics were given to suppress class II induction by intestinal bacteria.

The coaguligand caused no gastrointestinal damage, as expected because of the absence of clotting factors outside of the blood, but caused coagulopathies in occasional mice when administered at high dosage.

The findings described in this report demonstrate the therapeutic potential of targeting human coagulationinducing proteins to tumor vasculature. For clinical application, antibodies or other ligands are needed that bind to molecules that are present on the surface of vascular endothelial cells in solid tumors but absent from endothelial cells in normal tissues. Tumor endothelial markers could be induced directly by tumorderived angiogenesis factors (Folkman, 1985) or cytokines (Burrows et al., 1991; Ruco et al., 1990), or could relate to the rapid proliferation (Denekamp & Hobson, 1982) and migration (Folkman, 1985) of endothelial cells during neovascularization.

Several candidate antibodies have been described.

The antibody TEC-11, against endoglin is a particular example that binds selectively to human tumor endothelial cells.

Other antibodies include FB5, against endosialin (Rettig et al., 1992), E-9, against an endoglin-like molecule (Wang et al., 1993), BC-1, against a fibronectin isoform (Carnemolla et al., 1989) and TP-1 and TP-3, against an osteosarcoma-related antigen (Bruland et al., 1988). CD34 has been reported to be upregulated on migrating endothelial cells and on the abluminal processes of budding capillaries in tumors and fetal tissues (Schlingemann et al., 1990). The receptors for vascular endothelial cell growth factor (VEGF) become upregulated in tumor blood vessels (Plate et al., 1993; Brown et al., 1993) probably in response to hypoxia (Thieme et al., 1995), and selectively concentrate VEGF in tumor vessels (Dvorak et al., 1991).

The induction of tumor infarction by targeting coagulation-inducing proteins to these and other tumor endothelial cell markers is proposed as a valuable new approach to the treatment of solid tumors. The coupling of human (or humanized) antibodies to human coagulation proteins to produce wholly human

coaguligands is particularly contemplated, thus permitting repeated courses of treatment to be given to combat both the primary tumor and its metastases.

EXAMPLE IV SYNTHESIS OF TRUNCATED TISSUE FACTOR (tTF) CONSTRUCTS tTF is herein designated as the extracellular domain of the mature tissue factor protein (amino acid 1-219 of the mature protein; SEQ ID NO:23). SEQ ID NO:23 is encoded by, e.g., SEQ ID NO:22.

A. H6rtTF1 H6 Ala Met Ala[tTF]. The tTF complimentary DNA (cDNA) was prepared as follows: RNA from J-82 cells (human bladder carcinoma) was used for the cloning of tTF. Total RNA was isolated using the GlassMaxTM RNA microisolation reagent (Gibco BRL). The RNA was reverse transcribed to cDNA using the GeneAmp RNA PCR kit (Perkin Elmer). tTF cDNA was amplified using the same kit with the following two primers: 5' primer: 5' GTC ATG CCA TGG CCT CAG GCA CTA CAA (SEQ ID NO:1) 3' Primer: 5' TGA CAA GCT TAT TCT CTG AAT TCC CCC TTT CT (SEQ ID NO:2) The underlined sequences codes for the N-terminus of tTF. The rest of the sequence in the 5' primer is the restriction site for NcoI allowing the cloning of tTF into the expression vector. The sequence in the 3' primer is the HindIII site for cloning tTF into the expression vector. PCR amplification was performed as suggested by the manufacturer. Briefly, 75 yM dNTP; 0.6 zM primer, 1.5 mM MgC12 were used and 30 cycles of 30" at 950C, 30" at 550C and 30" at 720C were performed.

The E. coli expression vector H6 pQE-60 was used for expressing tTF (Lee et al., 1994). The PCR amplified tTF cDNA was inserted between the NcoI and Hind3 site. H6 pQE-60 has a built-in (His)6 encoding sequence such that the expressed protein has the sequence of (His)6 at the N terminus, which can be purified on a Ni-NTA column.

To purify tTF, tTF containing H6 pQE-60 DNA was transformed to E. coli TG-1 cells. The cells were grown to OD600 = 0.5 and IPTG was added to 30 HM to induce the tTF production. The cells were harvested after shaking for 18 h at 300C. The cell pellet was denatured in 6 M Gu-HCl and the lysate was loaded onto a Ni-NTA column (Qiagen). The bound tTF was washed with 6 M urea and tTF was refolded with a gradient of 6 M - 1 M urea at room temperature for 16 h. The column was washed with wash buffer (0.05 Na H2 P04, 0.3 M Nacl, 10% glycerol) and tTF was eluted with 0.2 M Imidozole in wash buffer. The eluted tTF was concentrated and loaded onto a G-75 column. tTF monomers were collected.

B. tTF Gly[tTF]. The GlytTF complimentary DNA (cDNA) was prepared the same way as described in the previous section except the 5' primer was replaced by the following primer in the PCR.

5' primer: 5' GTC ATG CCA TGG CCC TGG TGC CTC GTG CTT CTG GCA CTA CAA ATA CT (SEQ ID NO:3) The underlined sequence codes for the N-terminus of tTF. The remaining sequence encodes a restriction site for NcoI and a cleavage site for thrombin.

The H6 pQE60 expression vector and the procedure for protein purification is identical to that described above except that the final protein product was treated with thrombin to remove the H6 peptide. This was done by adding 1 part of thrombin (Sigma) to 500 parts of tTF (w/w), and the cleavage was carried out at room temperature for 18 h. Thrombin was removed from tTF by passage of the mixture through a Benzamidine Sepharose 6B thrombin affinity column (Pharmacia).

C. Cysteine-modified tTFS tTF constructs were modified with an N or C-terminal cysteine to allow for easier conjugation to derivatized antibody through a disulfide bond.

H6 C[tTF]. (His)6 Ala Met Ala Cys-[tTF1. The DNA was made as described in the previous section except that the 5' primer was replaced by the following primer in the PCR.

5' primer: 5' GTC ATG CCA TGG CCT GCT CAG GCA CTA CAA ATA CTG TG (SEQ ID NO:4) All of the procedures were the same as described above, except the N-terminal cys was protected with an exchangeable oxidizing/reducing reagent.

C[tTF]. Gly Ser Cys [tTF2-219]. The DNA was made as described in the previous section except that the 5' primer was replaced by the following primer in the PCR.

5' primer: 5' GTC ATG CCA TGG CCC TGG TGC CTC GTG GTT CTT GCG GCA CTA CAA ATA CT (SEQ ID NO:5) The vector construct and protein purification is the same as described for the (His)6 Ala Met Ala

Cys [tTF] construct, except that thrombin treatment was used to remove the (His)6 as described above.

H6 [tTF]C. (His)6 Ala Met Ala [tTF] Cys. The DNA was made the same way as described in the (His)6 AMA [tTF] sections, except that the 3' primer was replaced by the following primer.

3'primer:5'TGA CAA GCT TAG CAT TCT CTG AAT TCC CCC TTT CT (SEQ ID NO:6) The underlined sequence encodes the C-terminus of tTF. The rest of the sequence contains the HindIII restriction site for cloning tTF in to the expression vector.

All of the procedures are the same as described in the tTF section except that 10 mM p-ME was used in the 6 M Gu-HC1 denaturing solution and the C-terminal cysteine was protected with an exchangeable oxidizing/reducing reagent.

Other [tTF] Cys monomers, such as [tTF 1-220] Cys, [tTF 1-221] Cys and [tTF 1-222] Cys are also made (and conjugated) using the same methodology.

D. C Linker (tTF] The C Linker [tTF], Gly-Ser-Cys-(Gly)4-Ser-(Gly)4 Ser-(Gly)4-Ser-[tTF], was also constructed. The cDNA was made using a two step PCR procedure as follows: PCR 1: amplification of linker DNA cDNA encoding the NcoI site, the thrombin cleavage site, cysteine, linker and the N-terminus of tTF was amplified using the following primers: 5' primer: 5' GTC ATG CCA TGG CCC TGG TGC CTC GTG GTT GCG GA GGC GGT GGA TCA GGC (SEQ ID NO:7) 3' primer: 5' AGT ATT TGT AGT GCC TGA GGA TCC GCC ACC TCC ACT (SEQ ID NO:8) The underlined sequences encode the linker peptide.

The DNA template used in the PCR was double strand DNA encoding the following linker.

Sequence: GGA GGC GGT GGA TCA GGC GGT GGA GGT AGT GGA GGT GGC GGA TCC (SEQ ID NO:9) The same PCR conditions were used as described in the tTF section. The 95 b.p. amplified product was linked to tTF DNA in the PCR2.

PCR 2: Linking the Cys-linker DNA to tTF DNA. DNA templates used in the PCR were two overlapping DNA: The 95 b.p. DNA from PCR 1 as described above and tTF DNA.

The primers used were the following: 5' primer: 5' GTC ATG CCA TGG CCC TG (SEQ ID'NO:10) 3' primer: 5' TGA CAA GCT TAT TCT CTG AAT TCC CCC TTT CT (SEQ ID NO:ll) The final PCR product of 740 b.p. was digested with NcoI and HindIII and inserted into the H6 pQE 60 as described in the tTF section.

The vector constructs and protein purification procedures are all the same as described in the C[tTF] section.

EXAMPLE V SYNTHESIS OF DIMERIC TISSUE FACTOR The inventors' reasoned that tissue factor dimers may be more potent than monomers at initiating coagulation. It is possible that native tissue factor on the surface of J82 bladder carcinoma cells may exist as a dimer (Fair et al., 1987). The binding of one factor VII or VIIa molecule to one tissue factor molecule may also facilitate the binding of another factor VII or VIIa to another tissue factor (Fair et al., 1987; Bach et al., 1986). Furthermore, tissue factor shows structural homology to members of the cytokine receptor family (Edgington et al., 1991) some of which dimerize to form active receptors (Davies and Wlodawer, 1995). The inventors therefore synthesized TF dimers, as follows.

A. (tTFi Linker (tTFi.

The Gly [tTF] Linker [tTF] with the structure Gly[tTF] (Gly)4 Ser (Gly)4 Ser (Gly)4 Ser [tTF] was made.

Two pieces of DNA were PCR amplified separately and were ligated and inserted into the vector as follows: PCR 1: Preparation of tTF and the 5' half of the linker DNA. The primer sequences in the PCR are as follows: 5' primer: 5' GTC ATG CCA TGG CCC TGG TGC CTC GTG GTT CTT GCG GCA CTA CAA ATA CT (SEQ ID NO:12) 3' primer: 5' CGC GGA TCC ACC GCC ACC AGA TCC ACC GCC TCC TTC TCT GAA TTC CCC TTT CT (SEQ ID NO:13) Gly[tTF] DNA was used as the DNA template. Further PCR conditions were as described in the tTF section.

PCR 2: Preparation of the 3' half of the linker DNA and tTF DNA. The primer sequences in the PCR were as

follows: 5' primer: 5' CGC GGA TCC GGC GGT GGA GGC TCT TCA GGC ACT ACA AAT ACT GT (SEQ ID NO:14) 3' primer: 5' TGA CAA GCT TAT TCT CTG AAT TCC CCT TTC T (SEQ ID NO:15) tTF DNA was used as the template in the PCR. The product from PCR 1 was digested with NcoI and BamH. The product from PCR 2 was digested with HindIII and BamHI.

The digested PCR1 and PCR2 DNA were ligated with NcoI and HindIII-digested H6 pQE 60 DNA.

For the vector constructs and protein purification, the procedures were the same as described in the Gly [tTF] section.

B. Cvs [tTFl Linker (tTFi The Cys [tTF] Linker [tTF] with the structure Ser Gly Cys [tTF 2-219] (Gly)4 Ser (Gly)4 Ser(Gly)4 Ser [tTF] was also constructed. DNA was made by PCR using the following primers were used: 5' primer: 5' GTC ATG CCA TGG CCC TGG TGC CTC GTG GTT CTT GCG GCA CTA CAA ATA CT (SEQ ID NO:16) 3' primer: 5' TGA CAA GCT TAT TCT CTG AAT TCC CCT TTC T (SEQ ID NO:17) [tTF] linker [tTF] DNA was used as the template.

The remaining PCR conditions were the same as described in the tTF section. The vector constructs and protein purification were all as described in the purification of H6C[tTF].

C. rtTF] Linker [tTFicvs The [tTF] Linker [tTF]cys dimer with the protein structure [tTF] (Gly)4 Ser (Gly)4 Ser (Gly)4 Ser [tTF] Cys was also made. The DNA was made by PCR using the following primers: 5' primer: 5' GTC ATG CCA TGG CCC TGG TGC CTC GTG GTT GCA CTA CAA ATA CT (SEQ ID NO:18) 3'primer:5' TGA CAA GCT TAG CAT TCT CTG AAT TCC CCT TTC T (SEQ ID NO:19).

[tTF] linker [tTF] DNA was used as the template.

The remaining PCR conditions were the same as described in the tTF section. The vector constructs and protein purification were again performed as described in the purification of [tTF]cys section.

D. Chemically Contuaated Dimers [tTF] Cys monomer are conjugated chemically to form [tTF] Cys-Cys [tTF] dimers. This is done by adding an equal molar amount of DTT to the protected [tTF] Cys at room temperature for 1 hr to deprotect and expose the cysteine at the C-terminus of [tTF] Cys. An equal molar amount of protected [tTF] Cys is added to the DTT/[tTF] Cys mixture and the incubation is continued for 18 h at room temperature. The dimers are purified on a G-75 gel filtration column.

The Cys [tTF] monomer is conjugated chemically to form dimers using the same method.

EXAMPLE VI SYNTHESIS OF TISSUE FACTOR MtJTANTS Two tTF mutants are described that lack the capacity to convert tTF-bound factor VII to factor VIIa. There is 300-fold less factor VIIa in the plasma compared with factor VII (Morrissey et al., 1993). Therefore, circulating mutant tTF should be less able to initiate coagulation and hence exhibit very low toxicity. In coaguligands, once the mutant tTF has localized through the attached antibody to the tumor site, Factor VIIa will be injected to exchange with the tTF-bound Factor VII.

The mutants are active in the presence of factor VIIa.

A. [tTF1 G164A The ',[tTF]G164A" has the mutant protein structure with the amino acid 164 (Gly) of tTF being replaced by Ala. The Chameleon double-stranded site directed mutagenesis kit (Stratagene) is used for generating the mutant. The DNA template is Gly[tTF] DNA and the sequence of the mutagenizing primer is: 5' CAA GTT CAG CCA AGA AAAC (SEQ ID NO:20) The vector constructs and protein purification procedures described above are used in the purification of Gly[tTF]-.

B. rtTFl W158R S162A The [tTF]W158R S162A is a double mutant in which amino acid 158 (Trp) of tTF is replaced by Arg and amino acid 162 (Ser) is replaced by Ala. The same mutagenizing method is used as described for [tTF] G164A. The mutagenizing primer is: 5' ACA CTT TAT TAT CGG AAA TCT TCA GCT TCA GGA AAG (SEQ ID NO:21) The foregoing vector constructs and protein purification procedures are used for purifying Gly[tTF].

EXAMPLE VII SYNTHESIS OF TISSUE FACTOR CONJUGATES A. Chemical Derivatization and Antibodv Connuaation Antibody tTF conjugates were synthesized by the linkage of chemically derivatized antibody to

chemically derivatized tTF via a disulfide bond (as exemplified in FIG. 5).

Antibody was reacted with a 5-fold molar excess of succinimidyl .oxycarbonyl -a-methyl a- (2- pyridyldithio) toluene (SMPT) for 1 hour at room temperature to yield a derivatized antibody with an average of 2 pyridyldisulphide groups per antibody molecule. Derivatized antibody was purified by gel permeation chromatography.

A 2.5-fold molar excess of tTF over antibody was reacted with a 45-fold molar excess of 2-iminothiolane (2IT) for 1 hour at room temperature to yield tTF with an average of 1.5 sulfhydryl groups per tTF molecule.

Derivatized tTF was also purified by gel permeation chromatography and immediately mixed with the derivatized antibody.

The mixture was left to react for 72 hours at room temperature and then applied to a Sephacryl S-300 column to separate the antibody-tTF conjugate from free tTF and released pyridine-2-thione. The conjugate was separated from free antibody by affinity chromatography on a antitTF column. The predominant molecular species of the final conjugate product was the singly substituted antibody-tTF conjugate (Mr approx. 176,000) with lesser amounts of multiply substituted conjugates (Mr > approx.

202,000) as assessed by SDS-PAGE.

B. Coniuqation of Cvsteine-Modified tTF to Derivatized Antibody Antibody-C[TF] and [tTF]C conjugates were synthesized by direct coupling of cysteine-modified tTF to chemically derivatized antibody via a disulfide bond (as exemplified in FIG. 5).

Antibody was reacted with a 12-fold molar excess of 2IT for 1 hour at room temperature to yield derivatized antibody with an average of 1.5 sulfhydryl groups per antibody molecule. Derivatized antibody was purified by gel permeation chromatography and immediately mixed with a 2-fold molar excess of cysteine-modified tTF. The mixture was left to react for 24 hours at room temperature and then the conjugate was purified by gel permeation and affinity chromatography as described above.

The predominant molecular species of the final conjugate was the singly substituted conjugate (Mr approx. 176,000) with lesser amounts of multiple substituted conjugates (Mr > approx. 202,000) as assessed by SDS-PAGE.

C. Conlugation of Cysteine-Modified tTF to Fab' Fragments Antibody Fab'-C[tTF] and [tTF]C conjugates are prepared. Such conjugates may be more potent in vivo because they should remain on the cell surface for longer than bivalent conjugates due to their limited internalization capacity. Fab' fragments are mixed with a 2-fold molar excess of cysteine-modified tTF for 24 hours and then the conjugate purified by gel permeation and affinity chromatography as described above.

D. Clotting Activity of tTF Coniuaates tTF conjugates were prepared with the B21-2 monoclonal antibody which binds to Class II antigens expressed on the surface to A20 cells. The conjugates were prepared with chemically derivatized tTF and cysteine-modified tTF and the ability of the conjugates to clot mouse plasma in CaC12 was determined after their binding to the surface of A20 cells.

Both B21-2 conjugates shortened the clotting time of mouse plasma in CaC12 (control) in a dose-dependent manner. The tTF conjugates displayed a similar enhancement in coagulation as occurred when tTF was tethered to the surface of A20 cells with the bispecific antibody B21-2/10H10 (FIG. 6).

E. Anti-tumor Cell tTF Coniuaates It has already been established that when tTF is targeted to tumor vascular endothelial cells it induces coagulation within the tumor vessels (Examples I through III). The inventors' contemplated that coagulation would be induced in tumor vessels if tTF was targeted to the surface of tumor cells.

Three anti-tumor cell antibodies, KS1/4, D612, and XMMCO-791, were conjugated to tTF as described in the "Preparation of tTF conjugates" section above. KS1/4 was obtained from Dr. R. Reisfeld at the Scripps Research Institute, Department of Immunology, La Jolla, California, and is also described in U.S. Patent 4,975,369; D612 was obtained from Dr. J. Schlom at the NCI, Laboratory of Tumor Immunology and Biology, Bethesda, Maryland, is described in U.S. Patent 5,183,756 and can be obtained from culture

supernatants from the ATCC hybridoma cell line Accession No. HB 9796; XMMCO-791 was purified from tissue culture supernatant from the hybridoma cell line purchased from the ATCC.

The human colon carcinoma cell line Widr was used as a target cell for KS1/4. Widr cells were purchased from the ATCC and were maintained in DMEM supplemented with 10% (v/v) fetal calf serum, L-glutamine and antibiotics in an atmosphere of 10% (v/v) CO2 in air. The human colon carcinoma cell line LS147T was used as a target cell for D612. LS147T cells were purchased from the ATCC and were maintained in RPMI supplemented with 10% (v/v) fetal calf serum, L-glutamine and antibiotics in an atmosphere of 5% (v/v) CO2 in air. The human non small cell lung cancer cell line H460 was used as a target cell for XMMCO-791. H460 cells were obtained from Dr.Adi Gazdar, Simmons Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas and were maintained in DMEM supplemented with 10% (v/v) fetal calf serum, L-glutamine and antibiotics in an atmosphere of 10% (v/v) CO2 in air. All three cell lines grew as adherent monolayers.

The conjugates were tested for their ability to enhance the clotting time of mouse plasma in CaC12 when bound to tumor cells expressing the relevant target antigens. Tumor cells were removed from tissue culture flasks with 0.05% (w/v) EDTA in PBS. The cells were preincubated with TF9-6B4 and TF8-5G9 antibodies to neutralize any native tissue factor activity (Morrisey et. al., 1988) and then the coagulation assay was performed as described for A20 cells.

When bound to their target cell line, all three conjugates shortened the clotting time of mouse plasma in CaC12 (control) in a dose-dependent manner (FIG. 7), indicating that coagulation was accelerated at the surface of tumor cells when tTF was targeted to the cell surface.

EXAMPLE VIII SYNTHESIS OF TISSUE FACTOR PRODRUGS Exemplary tTF prodrugs have the following structures: tTFI-219 (X)nl (Y)n2 Z Ligand, where tTF1-219 represents TF minus the cytosolic and transmembrane domains; X represents a hydrophobic transmembrane domain nl amino acids (AA) in length (1-20 AA); Y represents a hydrophilic protease recognition sequence of n2 AA in length (sufficient AA to ensure appropriate protease recognition); Z represents a disulfide thioester or other linking group such as (Cys)1-2 Ligand represents an antibody or other targeting moiety recognizing tumorcells, tumor EC, connective tissue (stroma) or basal lamina markers.

The tTF prodrug is contemplated for injection intravenously allowing it to localize to diseased tissue (i.e. tumor). Once localized in the diseased tissue, endogenous proteases (i.e., metalloproteinases, thrombin, factor Xa, factor VIIa, factor IXa, plasmin) will cleave the hydrophilic protease recognition sequence from the prodrug which will allow the hydrophobic transmembrane sequence to insert into a local cell membrane. Once the tail has inserted into the membrane, the tTF will regain its coagulation-inducing properties resulting in clot formation in the vasculature of the diseased tissue.

EXAMPLE IX SYNTHESIS OF COAGULATION FACTORS LACKING THE Gla MODIFICATION The vitamin-K-dependent coagulation factors (Factor II/IIa, Factor VII/VIIa, Factor IX/IXa and Factor X/Xa) lacking the Gla (y-carboxyglutamic acid) modification are contemplated to be useful for the formation of coaguligands. Coagulation factors lacking the Gla modification are poor coagulants because the unmodified factors associate inefficiently with lipid membranes: targeting the factor via a ligand to the vasculature of tumors or other sites should bring the factor back into proximity to the cell surface and enable coagulation to proceed in that site.

"Gla" is made post-translationally by modifying the existing Glu (Glutamic acid) residues. Vitamin-Kdependent coagulation factors (Factor II/IIa, Factor VII/VIIa, Factor IX/IXa and Factor X/Xa) lacking the Gla modification may be made by expressing the genes that encode them in a host, such as bacteria, that does not modify Glu to Gla. The DNA sequences encoding each of Factor II/IIa, Factor VII/VIIa, Factor IX/IXa and Factor X/Xa are included herein as SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26 and SEQ ID NO:27, respectively. Procaryotic expression is therefore straightforward.

Such Gla-lacking factors may also be made by mutating any of the sequences described above (SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26 and SEQ ID NO:27) to change the corresponding Glu residues to another amino acid before expressing the genes, this time in virtually any host cell. The codon to be changed is the GAG codon (GAA also encodes Glu and is to be avoided). Using Factor VII as an example, the Gla "domain" is located generally in the 216-325 region. The first Gla-encoding triplet occurs at 231 of SEQ ID NO:25, and the last extends through 318 of SEQ ID NO:25. The GAG codons may readily be

changed using molecular biological techniques.

FIG. 8 shows that the Gla domains of each of the above vitamin-K-dependent coagulation factors lie in an analogous region. Therefore, mutation of the so-called "corresponding" Glu residues in any one of SEQ ID NO:24, SEQ ID NO:26 and SEQ ID NO:27 will also be straightforward.

The following Table of codons is provided to enable ready mutation choices to be made in modifying a given Gla-encoding codon or sequence.

Amino Acids Codons Alanine Ala A GCA GCC GCG GCU Cysteine Cys C UGC UGU Aspartic Asp D GAC GAU acid Glutamic Glu E GAA GAG acid Phenyla- Phe F UUC WW lanine Glycine Gly G GGA GGC GGG GGU Histidine His H CAC CAU Isoleucine Ile I AUA AUC AUU Lysine Lys K AAA AAG Leucine Leu L UUA WG CUA CUC CUG CW Methionine Met M AUG Asparagine Asn N AAC AAU Proline Pro P CCA CCC CCG CCU Glutamine Gln Q CAA CAG Arginine Arg R AGA AGG CGA CGC CGG CGU Serine Ser S AGC AGU UCA UCC UCG UCU Threonine Thr T ACA ACC ACG ACU Valine Val V GUA GUC GUG GUU Tryptophan Trp W UGG Tyrosine Tyr Y UAC UAU Site-specific mutagenesis is the technique contemplated for use in the preparation of individual vitamin-K-dependent coagulation factors lacking the Gla modification, through specific mutagenesis of the underlying DNA and the introduction of one or more nucleotide sequence changes into the DNA.

Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed.

Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by publications such as Adelman et al. (1983) and by the TF mutant studies described above. The technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (Messing et al., 1981). These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a singlestranded vector or melting apart the two strands of a double stranded vector which includes within its sequence a DNA sequence which encodes the vitamin-K-dependent coagulation factor. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example by the method of Crea et al.

(1978). This primer is then annealed with the singlestranded vector, and subjected to DNA polymerizing enzymes such as E. coli polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as E. coli cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

EXAMPLE X FURTHER ANT I - TUMOR VASCULATURE ANTIBODIES This example describes the generation of antibodies directed against tumor-derived endothelial cell "binding factors', for use in distinguishing between tumor vasculature and the vasculature of normal tissues.

Particularly described is the generation of antibodies directed against vascular permeability factor (VPF), also termed vascular endothelial cell growth factor (VEGF), and against bFGF (basic fibroblast growth factor).

For further details concerning FGF one may refer to Gomez-Pinilla and Cotman (1992); Nishikawa et al. (1992), that describe the localization of basic fibroblast growth factor; Xu et al. (1992), that relates to the expression and immunochemical analysis of FGF; Reilly et al. (1989), that concerns monoclonal antibodies;

Dixon et al. (1989), that relates to FGF detection and characterization; Matsuzaki et al. (1989), that concerns monoclonal antibodies against heparin-binding growth factor; and Herblin and. Gross (1992), that discuss the binding sites for bFGF on solid tumors associated with the vasculature.

In the present studies, rabbits were hyperimmunized with N-terminal peptides of human VEGF, mouse VEGF, guinea pig VEGF, human bFGF, mouse bFGF or guinea pig bFGF coupled to tuberculin (purified protein derivative, PPD) or thyroglobulin carriers. The peptides were 25 to 26 amino acids in length and were synthesized on a peptide synthesizer with cysteine as the C-terminal residue. Antisera were affinity purified on columns of the peptides coupled to Sephraose matrices.

Antibodies to VEGF were identified by ELISA and by their staining patterns on frozen sections of guinea pig tumors and normal tissues. Polyclonal antibodies to guinea pig VEGF and human VEGF reacted with the majority of vascular endothelial cells on frozen sections of guinea pig L10 tumors and a variety of human tumors (parotid, ovarian, mammary carcinomas) respectively. The anti-human VEGF antibody stained mesangial cells surrounding the endothelial cells in normal human kidney glomerulae and endothelial cells in the liver, but did not stain blood vessels in normal human stomach, leg muscle and spleen. The anti-guinea pig VEGF antibody did not stain endothelial cells in any normal tissues, including kidney, brain, spleen, heart, seminal vesicle, lung, large intestine, thymus, prostrate, liver, testicle and skeletal muscle.

Polyclonal antibodies to human FGF stained endothelial cells in parotid and ovarian carcinomas, but not those in mammary carcinomas. Anti-human FGF antibodies stained glomerular endothelial cells in human kidney, but not endothelial cells in normal stomach, leg muscle and spleen.

Monoclonal antibodies to guinea pig VEGF, human VEGF and guinea pig bFGF were prepared by immunizing BALB/c mice with the N-terminal sequence peptides (with cysteine at the C-terminus of the peptide) coupled to PPD or to thyroglobulin. The synthetic peptides immunogens of defined sequence are shown below and are represented by SEQ ID NO:30, SEQ ID NO:31 AND SEQ ID NO:32, respectively: guinea pig VEGF A P M A E G E Q K P R E V V K F M D V Y K R S Y C.

human VEGF A P M A E G G G Q N H H E V V K F M D V Y Q R S Y C guinea pig bFGF M A A G S I T T L P A L P E G G D G G A F A P G C The peptides were conjugated to thyroglobulin or to PPD by derivatizing the thyroglobulin with succimidyl 4 (N-maleimidomethyl) cyclohexane-l-carboxylate (SMCC) and reacting the derivative with the peptide. This yields a conjugate having one or more peptide sequences linked via a thioether bond to thyroglobulin.

Specifically, the generation of monoclonal antibodies against the above sequences was achieved using the following procedure: BALB/e mice were immunized by serial injections with peptide-PPD or peptidethyroglobulin into several sites. Four or five days after the last injection, the spleens were removed and splenocytes were fused with P3xG3Ag8.653 myeloma cells using polyethyleneglycol according to the procedures published in Morrow, et al. (1991).

Individual hybridoma supernatants were screened as follows: First screen: ELISA on peptide-thyroglobulin-coated plates.

Second screen: ELISA on cysteine linked via SMCC to thyroglobulin.

Third screen: Indirect immunoperoxidase staining of frozen sections of guinea pig line 10 tumor or human parotid carcinoma.

Fourth screen: Indirect immunoperoxidase staining of frozen sections of miscellaneous malignant and normal guinea pig and human tissues.

Antibodies were selected that bound to peptidethyroglobulin but not to cysteine-thyroglobulin, and which bound to endothelial cells in malignant tumors more strongly than they did to endothelial cells in normal tissues (Table VIII).

TABLE VIII Reactivity of Monoclonal Antibodies EMI198.1

MoAB	Immunoge	Class	Reactivity	Tumor
n+	with	Tumor	Reactivity	

Endothelium Pattern*
g. pig human
GV14 gp VEGF IgM + + BV + some tumor cells
GV35 gp VEGF IgM # # Tumor cells weak on BV
GV39 gp VEGF IgM + + BV and some tumor cells
GV59 gp VEGF IgM + + BV and some tumor cells
GV97 gp VEGF IgM + + BV weak on tumor cells
HV55 hu VEGF IgG ? + Basement membrane, some BV
GF67 gp FGF IgM + + BV and tumor cells
GF82 gp FGF IgM + + BV and tumor cells

* BV = blood vessels + gp = guinea pig hu = human A. GV97 Obtaining of Human and Guinea Pig Tissue Sections The GV97 antibody against guinea pig VEGF N-terminus bound to endothelial cells in miscellaneous human malignant (Table IX) and normal (Table X) tissues.

Binding to endothelial cells in malignant tumors tended to exceed that to endothelial cells in normal tissues.

The staining of endothelial cells in guinea pig tumor (line 10 hepatocellular carcinoma) and normal tissues was similar in distribution and intensity to that observed with human tissues (Table XI).

In the Tables, + indicates a positive, as opposed to a negative, result. The numbers 2+, 3+ and 4+ refer to a positive signal of increasing strength, as is routinely understood in this field of study.

TABLE IX. Anti-GPVEGF on Human Tumors EMI200.1

Purified GV97
1ug/ml
20 0.5 GV97 GV1 GV3 GV59
Tumor TISSUE 10ug/ml 5ug/ml or
ug/ml ug/ml supt. 4 9 supt.

2ug/ml
DIGESTIVE TRACT
92-01-A073 2+ 1+ +/- -ve 4+ 4+
esophagus carcinoma
M4 Parotid 4+
87-07-A134 3+ 2+ +/- -ve 3+ 4+
Parotid carcinoma
M5 Parotid 4+
88-04-A010 1-2+ 1+ -ve -ve 1-3+
parotid adenoca.

90-11-B319 Adeno. 3-4+ 3 Ca. of colon to 4+
liver
94-02-B021C 3-4+ 3 Adenocarcinoma of 4+
colon
93-10-A333 Adeno. 4+ 2-4+ 1-4+ -ve-1+ 4+ 3+
Ca. of colon with
normal
TABLE IX continued... EMI201.1

Purified GV97
1ug/ml
20 0.5 GV97 GV1 GV3 GV59
Tumor TISSUE 10ug/ml 5ug/ml or
ug/ml ug/ml supt. 4 9 supt.

2ug/ml
93-02-B004 4+ 3-4+ 2-4+ 1-2+ 3-4+ 2 Villous and 3+
Adenomatous polyp
of colon
93-02-A130 3+ 2+ +/--1+ -ve 4+ 4+ 3-4+
Leiomyosarcoma in
colon
93-02-B020 4+ 2+ 2-3+ -ve- 1- 4+
Gastric Ca. 1+ 2+
93-04-A221 3-4+ 2-3+ 1-2+ -ve- 4+ 4+
Pancreas Adenoca. 0.5+
94-04-A390 rectum 4+ 3+ 1-2+ 1+ 3+
adenoca.

93-12-A160 tongue 1-2+ +/- -ve- -ve 3+ 3+
carcionmaadenoca.

101-84a Stomach 3+ 2+ -ve-1+ -ve most 3+
signet ring Ca. 1-2+
(101-84b pair) bit a
few
3-4+
90-05-A172 4+ 3+ 1-2+ -ve- -ve 3+
Stomach Adenoca. 1+
TABLE IX continued... EMI202.1

Purified GV97
1ug/ml
20 0.5 GV97 GV1 GV3 GV59
Tumor TISSUE 10ug/ml 5ug/ml or
ug/ml ug/ml supt. 4 9 supt.

2ug/ml
REPRODUCTIVE TRACT
91-10-A115 Squam. 1-4+ 1-3+ 1-2+ 1-2+ 1-4+ 1-3+
cell Ca. of vulva
93-03-A343 +/- - +/- to +/- to +/- 3- 3-4+
Prostate Adenoca. 3-4+ 2-3+ 1-2+ 4+
MUSCLE
IMMUNE SYSTEM
URINARY SYSTEM

93-10-B002 Renal 2+ 3+
cell Ca.

90-01-A225 Renal 4+ 4+ 3-4+ of 1-3+ 3-4+ 3+ 3-4+
cell Ca. most of
some
93-01-A257 3-4+ 2-3+ 1-2+ +/- 2-3+ 2-3+
Transit. cell Ca.

of bladder
TABLE IX continued... EMI203.1

Purified GV97
1ug/ml
20 0.5 GV97 GV1 GV3 GV59
Tumor TISSUE 10ug/ml 5ug/ml or
ug/ml ug/ml supt. 4 9 supt.

2ug/ml
ENDOCRINE SYSTEM
94-01-A246 4+ 4+ 3-4+ 3+ 4+ 3-4+
Pheochromocytoma
of adrenal
93-11-A074 3-4+ 3-4+ 2-3+ 1+ 3-4+ 4+
Adrenal Cort. Ca.

RESPIRATORY SYSTEM
93-08-N009 Lung 3-4+ 3- 3-4+
Adenoca. 4+
92-10-A316 Sq. 4+ 3-4+ 1-2+ -ve- 4+ 4+
cell lung Ca. 0.5+
03-05-A065 Lung 4+ 3-4+ -ve-1+ 1+ 3+ 3+
adenoca.

CENTRAL NERVOUS SYSTEM
94-01-A299 malig. 4+ 4+ 4+ 3-4+ 4+ 3-4+
metast. schwanoma
to Lymph node
92-10-A139 4+ 3-4+ 2-3+ 1-2+ 4+ 3-4+
Meningioma
TABLE IX continued... EMI204.1

Purified GV97
1ug/ml
20 0.5 GV97 GV1 GV3 GV59
Tumor TISSUE 10ug/ml 5ug/ml or
ug/ml ug/ml supt. 4 9 supt.

2ug/ml
91-12-A013 4+ 2-3+ -ve-3+ +/- 4+ 3+
Meningioma
93-03-A361 4+ 4+ 3+ 2+ 4+ 3+
Atypical
meningioma

INTEGUMENTARY SYSTEM
94-04-V037 Skin -ve to -ve to -ve to -ve 2- 2-3+
Sq. cell Ca. 4+ 3+ 1+ 3+
w/normal
89-02-225 leg 4+ 3-4+ 1+ 1+ 4+ 2+
sarcoma
MISC. TUMORS
TABLE X. Anti-GPVEGF on Human Normal Tissues EMI205.1

Purified GV97
1ug/ml or GV97 GV1 GV59
Tumor TISSUE 20ug/ml 10ug/ml 5ug/ml 0.5ug/ml GV39
2ug/ml supt. 4 supt.

DIGESTIVE SYSTEM
91-01-A128 3+ 2+ 1+ -ve 2-3+ 2-3+
Bladder wl
cysitis
94-02-B020 2-3+ 2-3+
uninvolved colon
92-01-A292 N. 4+ 4+ 4+ 3-4+ 4+ 3-4+
Colon
93-10-A116 N. Z-4+ 1-4+ 1.3+ -ve-2+ -ve 3-4+ 2-3+ 3-4+
Colon
90-06-A116 N. 3+ of 2+
colon many
93-02-A350 N. 3-4+ 3+ 1+ +/- 4+ 4+
esophagus
93-05-A503 N. 4+ 4+
Ileum
TABLE X continued... EMI206.1

Purified GV97
1ug/ml or GV97 GV1 GV59
Tumor TISSUE 20 ug/ml 10ug/ml 5ug/ml 0.5 ug/ml GV39
2ug/ml supt 4 supt.

94-03-A244 N. 4+ 1-3+ -ve-1+ -ve 4+ 4+
Liver
90-02-B132 N. 1+ of a +/- -ve -ve -ve 1-3+ 2- 2-3+ 2-3+
Liver few 3+
94-01-A181 N. 1-4+ 1-3+ 1-3+ of a -ve 3-4+
Pancreas few
90-05-DOO8 N. 2-4+ 1-3+ +/- -ve 2-3 2-3+
Pancreas
93-05-A174 N. 2+ of a few 1-2+ of a 1+ of a few -ve -ve 3+ of 2-3+
Protid few a few
94-04-A391 N. 1-3+ -ve-2+ -ve -ve 3+
Small bowel
88-06-107 N. 3+ 2+ +/- -ve 3-4+ 3+
Stomach
101-84b N. 3@4+ in 2-3+ in +/- in main -ve in main 3+ 3-4+
Stomach main and main and 3- and 2+ in and 1+ in
(101-84a pair) periphery 4+ in periphery periphery
periphery
TABLE X continued... EMI207.1

Purified GV97

1ug/ml or GV97 GV1 GV59
Tumor TISSUE 20 ug/ml 10ug/ml 5ug/ml 0.5 ug/ml GV39
2ug/ml supt. 4 supt
90-11-B337 N. 2-3 +/--1+ -ve -ve 3+ 3+
Stomach
REPRODUCTIVE TRACT
93-04-AO41 N. 4+ 3+
Breast
94-02-A197 N. 4+ 3+
Breast
w/fibrocystic
change
93-02-A051 -ve-1+ -ve -ve -ve +/- +/@@2+
Breast
w/fibrocystic
change
93-02-A103 4+ 3+ 2+ 1+
Breast
w/fibrocyst.

change
92-11-A006 N. 2+ of 1-2+ of 0.5+ -ve -ve 1-2+ of 3+ of
ectocervix most most some most
TABLE X continued... EMI208.1

Purified GV97
1ug/ml or GV97 GV1 GV59
Tumor TISSUE 20 ug/ml 10ug/ml 5ug/ml 0.5 ug/ml GV39
2ug/ml supt. 4 supt.

91-03-A207 N. 2.5+ 1.5+ 1+ .5+ 2-3+
ectocervix
92-02-A139 N. 1+ in -ve in most -ve -ve -ve in -ve in
ovary w/corp. most but but 1 + in most but most
lusteum 2+ in -one area 3-4+ in bet 3-4
one area one area in one
area
93-06-A11B N. 1+ of a few -ve -ve -ve 3+
Prostate
93-11-A317d 3-4+ 2-3+ -ve-3+ -ve-1+ 3-4+ 3-4+
Prostate chip
93-02-A315 0.5-1+ 0.5+ -ve -ve 1+ 1.2+
Seminal Vesicle
92-04-A069 N. 1+ +/- +/-+/- 1-2+
testis
91-04-A117 1+ +/- -ve -ve +/-1+ 3.4+
Ureter
w/inflammation
TABLE X continued... EMI209.1

Purified GV97
1ug/ml or GV97 GV1 GV59
Tumor TISSUE 20 ug/ml 10ug/ml 5ug/ml 0.5 ug/ml GV39
2ug/ml supt. 4 supt.

MUSCLE
94-01-A065 N. 3-4+ 2+ +/- -ve 3-4+ 4+

Heart
91-07-D007 N. 1-4+ 1-3+ 1-2+ -ve -ve 1-3+ 1-3+
skeletal muscle
95-03-A395 N. 4+ 3-4+ 1-2+ 0.5-1+ 4+ 4+
Skeletal muscle
IMMUNE SYSTEM
90-01-A077 N. 2-3+ 2+ 1+ of some -ve -ve 2-3+ 3-4+
lymph node
90-08-AD22 N. most 1 + but most 0,5+ most -ve but most -ve but a 3+ 3+
lymph node a few 4+ but a few a few 2+ few 0.5-1+
2+
91-03-A057 N. 2+ 1+ +/- -ve 3-4+ 3-4+
lymph node
91-09-B017E 3+ 2+ +/--1+ -ve 2-3+ 2-3+
uninvolved lymph
node
TABLE X continued... EMI210.1

Purified GV97
1ug/ml or GV97 GV1 GV59
Tumor TISSUE 20 ug/ml 10ug/ml 5ug/ml 0.5 ug/ml GV39
2ug/ml supt. 4 supt.

93-07-A236 N. 3-4+ 3-4+ -ve-3+ -ve 2-4+
Spicen
93-07-252 N. 3+ 1+ +/- -ve 2-3+
spicen
ENDOCRINE SYSTEM
94-04-A252 N. 4+ 4+ 3-4+ 1-2+ 4+ 3+
adrenal w/
medulia and
cortex
93-05-A086 N most -ve a most -ve a -ve -ve 2-3+ 3-4+
Adrenal medulia few 1-2+ few 1-2+
92-03-A157 1+ +/- +/- -ve 4+ 4+
Hyperplastic
thyroid
91-03-B019 N. -ve-3+ -ve-2+ -ve-1+ -ve 2-3+ 2-3+
Thyroid
URINARY SYSTEM
TABLE X continued... EMI211.1

Purified GV97
1ug/ml or GV97 GV1 GV59
Tumor TISSUE 20 ug/ml 10ug/ml 5ug/ml 0.5 ug/ml GV39
2ug/ml supt. 4 supt.

93-09-AO48 N. 4+ 2-3+
Kidney
91-11-AO75 N. 4+ 3+ 2+ 1+ 4+ on 4+ on 4+ on
Kidney glomeruli glomer glomer glomerul
uli i
93-10-BOO1 N. 4+ 3+ +/- -ve 4+ on 4+ on 4+on
Kidney glomeruli glomer glomerul
uli i
INTEGUMENTARY SYSTEM
92-08-AO29 N. +/- to 4+ +/- to 3+ +/- to 1+ +/- 2+ 2+
Breast skin

89-02-257 4+ 3-4+ 2-3+ 1-2+ 1+ 3-4+
Cartiledge
marches 2SS
RESPIRATORY SYSTEM
93-05-A203 N. -ve-2+ -ve-1+ +/- -ve 2+ 3+
Lung
TABLE X continued... EMI212.1

Purified GV97
1ug/ml or GV97 GV1 GV59
Tumor TISSUE 20 ug/ml 10ug/ml 5ug/ml 0.5 ug/ml GV39
2ug/ml supt. 4 supt.

92-12-A263 N. 2-3+ 1-2+ w/ -ve -ve 2-3+
Bronchus w/ducts ducts
staining 3- staining 24+ 3+
TABLE XI. Staining Pattern of 9FT anti-VEGF by direct immunohistochemical staining on 6-8 week old GP tissues EMI213.1

Purified GV97
1ug/ml or
TISSUE 20 ug/ml 10ug/ml 5ug/ml 0.5 ug/ml 9F7 supt. 3F9 supt. 5F9 supt.

2ug/ml
DIGESTIVE SYSTEM
LIVER 2+ 1-2+ +/- +/- 1-2+ 1-2+
INTESTINE 4+ 3+ 2+ 1+ 4+m 4+m
lymphoid, lymphoid,
rest diff. rest diff.

than than
PANCREAS 1+ of
many and
3+ in
islands of
cells
SMALL 4+ of 2-3+ of 1-2 of +/- of many 3+ of 3+ of
INTESTINE many and many and many and and 4+ in some and some and
4+ in 4+ in 4+ in lymphoid, rest 4+ in 4+ in
lymphoid lymphoid, lymphoid, diff. than fVIII lymphoid lymphoid
rest diff. rest diff.

than fVIII than fVIII
TABLE XI continued... EMI214.1

Purified GV97
1ug/ml or
TISSUE 20 ug/ml 10ug/ml 5ug/ml 0.5 ug/ml 9F7 supt. 3F9 supt. 5F9 supt.

2ug/ml
STOMACH 3-4+ 1-2+ on +/- on +/- on most 3-4+ 3-4+
most most occasional 1+ (some (some
occasional occasional fVIII-ve) fVIII-ve)
3+ 2+
REPRODUCTIVE SYSTEM

TESTIS
MUSCLE AND INTEGUMENTARY SYSTEM
HEART -ve -ve -ve -ve 3-4+ 3-4+
(some (some
fVIII-ve) fVIII-ve)
MUSCLE
SKIN 1-2+ in 1+ in +/- in +/- in fatty 3+ 3+
fatty layer fatty fatty layer layer and 1and 3-4+ layer and and 3-4+ 2+ of a few
in cellular 3-4+ in of a few in cellular
layer cellular in cellular layer
layer layer
TABLE XI continued... EMI215.1

Purified GV97
1ug/ml or
TISSUE 20 ug/ml 10ug/ml 5ug/ml 0.5 ug/ml 9F7 supt. 3F9 supt. 5F9 supt.

2ug/ml
IMMUNE SYSTEM
SPLEEN 4+ 3+ 2+ -ve 4+ 4+
THYMUS
URINARY SYSTEM
KIDNEY glomeruli glomeruli glomeruli glomeruli 1-2+ glomeruli glomeruli
4+ 3-4+ 2-3+ 3-4+ 3-4+
ENDOCRINE SYSTEM
ADRENAL
RESPIRATORY SYSTEM
LUNG
NERVOUS SYSTEM
CEREBELLUM 4+ 2+ +/- of +/- of most 4+ 4+
most and and 1+ of a
1+ of a few
few
TABLE XI continued... EMI216.1

Purified GV97
1ug/ml or
TISSUE 20 ug/ml 10ug/ml 5ug/ml 0.5 ug/ml 9F7 supt. 3F9 supt. 5F9 supt.

2ug/ml
TUMORS
TUMOR 4+ 4+ 3-4+ 2-3+(2) 4+ 3+
B. Lack of Reactivity of GV97 With Soluble Human VEGF To identify antibodies that are specific for VEGF, the VEGF receptor (Flk-l) or VEGF bound (or complexed) to the receptor, an ELISA screening protocol was developed.

The procedure is as follows: Initially, a 96 well ELISA plate (round bottom) was coated (outside wells left blank) with 100 yl/well of FLK/seap at 10 yg/ml in sensitizing buffer. After overnight incubation, the plate was washed twice with PBS overnight at 40C. Next the FLK/Seap coated plate was blocked with 250 pl/well of PBS + CAH (5%) solution for 1 h at 370C. The blocking solution was removed and the plate was vigorously tapped on paper towels.

The blocked plates were then incubated with 100 yl/well of VEGF-165 (VEGF 165 aa form produced in yeast obtained from Dr. Ramakrishnan, University of Minnesota) at 2 yg/ml in binding plus 0.1 yg/ml heparin for 4 h at room temperature or overnight at 40C. The VEGF solution was collected and the plate washed 2 times with PBS-tween (0.10%). Next, 100 yl/well of hybridoma fusion supernatant was added to the wells and incubated for 1 h at 320C. Following this supernatant incubation, the plate was washed 3 times with PBS tween and then incubated with 100 81 well of secondary antibody (KPL, Gt anti-mouse IgG at 1:1000

in PBS tween + CAH (5%) for 1 hour at 370C.

Following secondary antibody incubation, the plates were washed 4 times with PBS tween, incubated with 100 yl/well of substrate (Substrate Sigma OPD dissolved in citrate buffer + H202) for 20 minutes and read at 490 nm on a Cambrige Technology Microplate Reader (Model 7520).

Wells with an absorbance above appropriate control wells were selected as positives and further characterized.

It was found that GV97 did not bind to recombinant VEGF-coated ELISA plates, nor did recombinant human VEGF bind to GV97 coated ELISA plates. Soluble recombinant human VEGF did not block the binding of 5 yg/ml GV97 to tumor endothelium in histological sections even when added at 50 ,ug/ml .

These data suggest that GV97 recognizes an epitope of VEGF that is concealed in recombinant human VEGF but which becomes accessible when VEGF binds to its receptor on endothelial cells.

C. GV97 Localization in Line 10-Bearing Guinea Pigs In contrast with staining data obtained from histological sections, GV97 antibody localized selectively to tumor endothelial cells after injection into line 10 tumor-bearing guinea pigs (Table XII).

Staining of endothelial cells in the tumor was moderately strong whereas staining of normal endothelium in miscellaneous organs was undetectable.

D. Anti-bFGF Selectively Bind to Tumor Endothelial Cells GV97 and GF82, which had been raised against guinea pig bFGF N-terminus, bound strongly to endothelial cells in frozen reactions of guinea pig line 10 tumor and to endothelial cells in two types of human malignant tumors (Table XIII). By contrast, relatively weak staining of endothelial cells in miscellaneous guinea pig normal tissues was observed.

TABLE XII. GV97 injected into tumor bearing GP EMI219.1

TISSUE	GV97 10 ug/ml	GV 97 20 ug/ml
serum volume		
injected		
DIGESTIVE SYSTEM		
LIVER	3+	-ve
INTESTINE	3+ possible	0.5-1+
of a few		
PANCREAS	+/- of many and possible	0.5-1+
2+ in islands of	of a few	
cells		
SMALL INTESTINE	2-3+ of many and	+/
4+ in lymphoid,		
rest diff. than		
fVIII		
STOMACH	1-2+ on most possibly	0.5+ of
occasional 3+ a	few	
REPRODUCTIVE SYSTEM		
TESTIS	+/	
MUSCLE AND INTEGUMENTARY SYSTEM		
HEART	-ve	-ve
MUSCLE	-ve	
SKIN	1+ in fatty	layer
and 3-4+ in		
cellular layer		
IMMUNE SYSTEM		
SPLEEN	3+ possibly a	few
1+		
THYMUS		
URINARY SYSTEM		

KINEY glomeruli 3-4+
ENDOCRINE SYSTEM
ADRENAL 4+ -ve
RESPIRATORY SYSTEM
LUNG 2+ -ve
NERVOUS SYSTEM
TABLE XII continued... EMI220.1

TISSUE GV97 10 ug/ml GV 97 20 ug/ml
serum volume
injected
CEREBELLUM 2+ -ve
TUMORS
TUMOR 4+ 2-3+
TABLE XIII. Anti-GP FGF Antibody Staining on GP Tissues EMI221.1

GP TISSUE ¦ GF 67 ¦ GF 82
DIGESTIVE SYSTEM
LIVER ND ND
INTESTINE +/- +/
PANCREAS 2-3+ 2+
SMALL INTESTINE +/
STOMACH ND ND
REPRODUCTIVE SYSTEM
TESTIS ND ND
MUSCLE AND INTEGUMENTARY SYSTEM
HEART 2-3+ 1+
MUSCLE +/- 1+
SKIN ND ND
IMUE SYSTEM
SPLEEN 3+ -ve
THYMUS
URINARY SYSTEM
KIDNEY 1-2+ -ve
ENDOCRINE SYSTEM
ADRENAL 1-2+ +/
RESPIRATORY SYSTEM
LUNG 1-2+ 2-3+
NERVOUS SYSTEM
CEREBELLUM 1+ -1+
TUMORS
LINE 1 TUMOR 4+ 4+
HUMAN TUMORS
PHEOCHROMO 4+ 4+
CYTOMA
SCHWANOMA 4+ 4+

EXAMPLE XI HUMAN TREATMENT PROTOCOLS This example is concerned with human treatment protocols using the bispecific binding and coagulating ligands of the invention.These ligands are contemplated for use in the clinical treatment of various human cancers and even other disorders, such as benign prostatic hyperplasia and rheumatoid arthritis, in which the intermediate or longer term arrest of blood flow would be advantageous.

The bispecific ligands are considered to be particularly useful tools in anti-tumor therapy. From the data presented herein, including the animal studies, and the knowledge in the art regarding treatment of Lymphoma (Glennie et al., 1988), T-Cell targeting (Nolan & Kennedy, 1990) and drug targeting (Paulus, 1985) appropriate doses and treatment regimens may be straightforwardly developed - Naturally, before wide-spread use, further animal studies and clinical trials will be conducted. The various elements of conducting a clinical trial, including patient treatment and monitoring, will be known to those of skill in the art

in light of the present disclosure. The following information is being presented as a general guideline for use in establishing such trials.

It is contemplated that patients chosen for the study would have failed to respond to at least one course of conventional therapy and had to have objectively measurable disease as determined by physical examination, laboratory techniques, or radiographic procedures. Where murine monoclonal antibody portions are employed, the patients should have no history of allergy to mouse immunoglobulin. Any chemotherapy should be stopped at least 2 weeks before entry into the study.

In regard to bispecific ligand administration, it is considered that certain advantages will be found in the use of an indwelling central venous catheter with a triple lumen port. The bispecific ligands should be filtered, for example, using a 0.22m filter, and diluted appropriately, such as with saline, to a final volume of 100 ml. Before use, the test sample should also be filtered in a similar manner, and its concentration assessed before and after filtration by determining the A280. The expected recovery should be within the range of 87 to 99%, and adjustments for protein loss can then be accounted for.

The bispecific ligands may be administered over a period of approximately 4-24 hours, with each patient receiving 2-4 number of infusions at 2-7 day intervals.

Administration can also be performed by a steady rate of infusion over a 7 day period. The infusion given at any dose level should be dependent upon any toxicity observed. Hence, if Grade II toxicity was reached after any single infusion, or at a particular period of time for a steady rate infusion, further doses should be withheld or the steady rate infusion stopped unless toxicity improved. Increasing doses of bispecific coagulating ligands should be administered to groups of patients until approximately 60% of patients showed unacceptable Grade III or IV toxicity in any category.

Doses that are 2/3 of this value could be defined as the safe dose.

Physical examination, tumor measurements, and laboratory tests should, of course, be performed before treatment and at intervals up to 1 month later.

Laboratory tests should include complete blood counts, serum creatinine, creatine kinase, electrolytes, urea, nitrogen, SGOT, bilirubin, albumin, and total serum protein. Serum samples taken up to 60 days after treatment should be evaluated by radioimmunoassay for the presence of the intact bispecific ligand or components thereof and antibodies against any or both portions of the ligand. Immunological analyses of sera, using any standard assay such as, for example, an ELISA or RIA, will allow the pharmacokinetics and clearance of the therapeutic agent to be evaluated.

To evaluate the anti-tumor responses, it is contemplated that the patients should be examined at 48 hours to 1 week and again at 30 days after the last infusion. When palpable disease was present, two perpendicular diameters of all masses should be measured daily during treatment, within 1 week after completion of therapy, and at 30 days. To measure nonpalpable disease, serial CT scans could be performed at l-cm intervals throughout the chest, abdomen, and pelvis at 48 hours to 1 week and again at 30 days. Tissue samples should also be evaluated histologically, and/or by flow cytometry, using biopsies from the disease sites or even blood or fluid samples if appropriate.

Clinical responses may be defined by acceptable measure. For example, a complete response may be defined by the disappearance of all measurable tumor 1 month after treatment. Whereas a partial response may be defined by a 50% or greater reduction of the sum of the products of perpendicular diameters of all evaluable tumor nodules 1 month after treatment, with no tumor sites showing enlargement. Similarly, a mixed response may be defined by a reduction of the product of perpendicular diameters of all measurable lesions by 50% or greater 1 month after treatment, with progression in one or more sites.

* * * All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure.

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which

are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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(iii) TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR THE SPECIFIC COAGULATION OF VASCULATURE (iv) NUMBER OF SEQUENCES: 32 (v) CORRESPONDENCE ADDRESS: (A) ADDRESSEE: Arnold, White & Durkee (B) STREET: P. O. Box 4433 (C) CITY: Houston (D) STATE: Texas (E) COUNTRY: USA (F) ZIP: 77210 (vi) COMPUTER READABLE FORM: (A) MEDIUM TYPE: Floppy disk (B) COMPUTER: IBM PC compatible (C) OPERATING SYSTEM: PC-DOS/MS-DOS, ASCII (vii) CURRENT APPLICATION DATA: (A) APPLICATION NUMBER: PCT/US95/07439 (B) FILING DATE: 07-JUN-1995 (C) CLASSIFICATION: Unknown (viii) PRIOR APPLICATION DATA: (A) APPLICATION NUMBER: US 08/273,567 (B) FILING DATE: ll-JUL-1994 (ix) ATTORNEY/AGENT INFORMATION: (A) NAME: PARKER, DAVID L.

(B) REGISTRATION NUMBER: 32,165 (C) REFERENCE/DOCKET NUMBER: UTFD433P- (x) TELECOMMUNICATION INFORMATION: (A) TELEPHONE: (512) 418-3000 (B) TELEFAX: (713) 789-2679 (C) TELEX: 79-0924 (2) INFORMATION FOR SEQ ID NO:1: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 27 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = DNA (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1: GTCATGCCAT GGCCTCAGGC ACTACAA 27 (2) INFORMATION FOR SEQ ID NO:2: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 32 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION:SEQ ID NO:2: TGACAAGCTT ATTCTCTGAA TTCCCCCTTT CT 32 (2) INFORMATION FOR SEQ ID NO:3: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 47 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = DNA (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3: GTCATGCCAT GGCCCTGGTG CCTCGTGCTT CTGGCACTAC AAATACT 47 (2) INFORMATION FOR SEQ ID NO:4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 38 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION:SEQ ID NO:4: GTCATGCCAT GGCCTGCTCA GGCACTACAA ATACTGTG 38 (2) INFORMATION FOR SEQ ID NO:5: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 50 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5: GTCATGCCAT GGCCCTGGTG CCTCGTGGTT CTTGCGGCAC TACAAATACT 50 (2) INFORMATION FOR SEQ ID NO:6: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 35 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = DNA (xi) SEQUENCE DESCRIPTION:SEQ ID NO:6: TGACAAGCTT AGCATTCTCT GAATTCCCCC TTTCT 35 (2) INFORMATION FOR SEQ ID NO:7: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 50 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7: GTCATGCCAT GGCCCTGGTG CCTCGTGGTT GCGGAGGCGG TGGATCAGGC 50 (2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 36 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION:SEQ ID NO:8: AGTATTTGTA GTGCCTGAGG ATCCGCCACC TCCACT 36 (2) INFORMATION FOR SEQ ID NO:9: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 45 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9: GGAGGCGGTG GATCAGGCGG TGGAGGTAGT GGAGGTGGCG GATCC 45 (2) INFORMATION FOR SEQ ID NO:10: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 17 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10: GTCATGCCAT

GGCCCTG 17 (2) INFORMATION FOR SEQ ID NO:11: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 32 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11: TGACAAGCTT ATTCTCTGAA TTCCCCCTTT CT 32 (2) INFORMATION FOR SEQ ID NO:12: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 50 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION:SEQ ID NO:12: GTCATGCCAT GGCCCTGGTG CCTCGTGGTT CTTGCGGCAC TACAAATACT 50 (2) INFORMATION FOR SEQ ID NO:13: (i) SEQUENCE CHARACTERSITICS: (A) LENGTH: 53 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13: CGCGGATCCA CCGCCACCAG ATCCACCGCC TCCTTCTCTG AATTCCCCTT TCT 53 (2) INFORMATION FOR SE ID NO:14: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 44 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = DNA (xi) SEQUENCE DESCRIPTION:SEQ ID NO:14: CGCGGATCCG GCGGTGGAGG CTCTTCAGGC ACTACAAATA CTGT 44 (2) INFORMATION FOR SEQ ID NO:15: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 31 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15: TGACAAGCTT ATTCTCTGAA TTCCCCTTTC T 31 (2) INFORMATION FOR SEQ ID NO:16: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 50 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION:SEQ ID NO:16: GTCATGCCAT GGCCCTGGTG CCTCGTGGTT CTTGCGGCAC TACAAATACT 50 (2) INFORMATION FOR SEQ ID NO:17: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 31 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17: TGACAAGCTT ATTCTCTGAA TTCCCCTTTC T 31 (2) INFORMATION FOR SEQ ID NO:18: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 44 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION:SEQ ID NO:18: GTCATGCCAT GGCCCTGGTG CCTCGTGGTT GCACTACAAA TACT 44 (2) INFORMATION FOR SEQ ID NO:19: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 34 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19: TGACAAGCTT AGCATTCTCT GAATTCCCCT TTCT 34 (2) INFORMATION FOR SEQ ID NO:20: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 19 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA (xi) SEQUENCE DESCRIPTION:SEQ ID NO:20: CAAGTT CAG C CAAGAAAAC 19 (2) INFORMATION FOR SEQ ID NO:21: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 36 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21: ACACTTTATT ATCGGAAATC TTCAGCTTCA GGAAAG 36 (2) INFORMATION FOR SEQ ID NO:22: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 657 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION:SEQ ID NO:22: TCAGGCACTA CAAATACTGT GGCAGCATAT AATTTAACTT GGAAATCAAC TAATTTCAAG 60 ACAATTTTGG AGTGGGAACC CAAACCCGTC AATCAAGTCT ACACTGTTCA AATAAGCACT 120 AAGTCAGGAG ATTGGAAAAG CAAATGCTTT TACACAACAG ACACAGAGTG TGACCTCACC 180 GACGAGATTG TGAAGGATGT GAAGCAGACG TACTTGGCAC GGGTCTTCTC CTACCCGGCA 240 GGGAATGTGG AGAGCACCGG TTCTGCTGGG GAGCCTCTGT ATGAGAACTC CCCAGAGTTC 300 ACACCTTACC TGGAGACAAA CCTCGGACAG CCAACAATTC AGAGTTTTGA ACAGGTGGGA 360 ACAAAAGTGA ATGTGACCGT AGAAGATGAA CGGACTTTAG TCAGAAGGAA CAACACTTTC 420 CTAAGCCTCC GGGATGTTTT TGGCAAGGAC TTAATTTATA CACTTTATTA TTGGAAATCT 480 TCAAGTTCAG GAAAGAAAAC AGCCAAAACA AACACTAATG AGTTTTTGAT TGATGTGGAT 540 AAAGGAGAAA ACTACTGTTT CAGTGTTCAA GCAGTGATTC CCTCCCGAAC AGTTAACCGG 600 AAGAGTACAG ACAGCCCGGT AGAGTGTATG GGCCAGGAGA AAGGGGAATT CAGAGAA 657 (2) INFORMATION FOR SQ ID NO:23: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 219 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: (D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23: Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr Asn Leu Thr Trp Lys Ser 1 5 10 15 Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln 20 25 30 Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys 35 40 45 Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val 50 55 60 Lys Asp Val Lys Gln Thr Tyr

Leu Ala Arg Val Phe Ser Tyr Pro Ala 65 70 75 80 Gly Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn 85 90 95 Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr 100 105 110 Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu 115 120 125 Asp Glu Arg Thr Leu Val Arg Arg Asn Asn Thr Phe Leu Ser Leu Arg 130 135 140 Asp Val Phe Gly Lys Asp Leu Ile Tyr Thr Leu Tyr Tyr Trp Lys Ser 145 150 155 160 Ser Ser Ser Gly Lys Lys Thr Ala Lys Thr Asn Thr Asn Glu Phe Leu 165 170 175 Ile Asp Val ASp Lys Gly Glu Asn Tyr Cys Phe Ser Val Gln Ala Val 180 185 190 Ile Pro Ser Arg Thr Val Asn Arg Lys Ser Thr Asp Ser Pro Val Glu 195 200 205 Cys Met Gly Gln Glu Lys Gly Glu Phe Arg Glu 210 215

(2) INFORMATION FOR SEQ ID NO:24: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1947 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION:SEQ ID NO:24: TGCAGCTGCC TGGCTGCCTG GCCCTGGCTG CCCTGTGTAG CCTTGTGCAC AGCCAGCATG 60 TGTTCCTGGC TCCTCAGCAA GCACGGTCGC TGCTCCAGCG GGTCCGGCGA GCCAACACCT 120 TCTTGGAGGA GGTGCGCAAG GGCAACCTAG AGCGAGAGTG CGTGGAGGAG ACGTGCAGCT 180 ACGAGGAGGC CTTCGAGGCT CTGGAGTCCT CCACGGCTAC GGATGTGTTC TGGGCCAAGT 240 ACACAGCTTG TGAGACAGCG AGGACGCCTC GAGATAAGCT TGCTGCATGT CTGGAAGGTA 300 ACTGTGCTGA GGGTCTGGGT ACGAACTACC GAGGGCATGT GAACATCACC CGGTCAGGCA 360 TTGAGTGCCA GCTATGGAGG AGTCGCTACC CACATAAGCC TGAAATCAAC TCCACTACCC 420 ATCCTGGGGC CGACCTACAG GAGAATTTCT GCCGCAACCC CGACAGCAGC AACACGGGAC 480 CCTGGTGCTA CACTACAGAC CCCACCGTGA GGAGGCAGGA ATGCAGCATC CCTGTCTGTG 540 GCCAGGATCA AGTCACTGTA GCGATGACTC CACGCTCCGA AGGCTCCAGT GTGAATCTGT 600 CACCTCCATT GGAGCAGTGT GTCCCTGATC GGGGGCAGCA GTACCAGGGG CGCCTGGCGG 660 TGACCACACA TGGGCTCCCC TGCCTGGCCT GGGCCAGCGC ACAGGCCAAG GCCCTGAGCA 720 AGCACCAGGA CTTCAACTCA GCTGTGCAGC TGGTGGAGAA CTTCTGCCGC AACCCAGACG 780 GGGATGAGGA GGGCGTGTGG TGCTATGTGG CCGGGAAGCC TGGCGACTTT GGGTACTGCG 840 ACCTCAACTA TTGTGAGGAG GCCGTGGAGG AGGAGACAGG AGATGGGCTG GATGAGGACT 900 CAGACAGGGC CATCGAAGGG CGTACCGCCA CAAGTGAGTA CCAGACTTTC TTCAATCCGA 960 GGACCTTTGG CTCGGGAGAG GCAGACTGTG GGCTGCGACC TCTGTTCGAG AAGAAGTCGC 1020 TGGAGGACAA AACCGAAAGA GAGCTCCTGG AATCCTACAT CGACGGGCGC ATTGTGGAGG 1080 GCTCGGATGC AGAGATCGGC ATGTCACCTT GGCAGGTGAT GCTTTTCCGG AAGAGTCCCC 1140 AGGAGCTGCT GTGTGGGGCC AGCCTCATCA GTGACCGCTG GGTCCTCACC GCCGCCCACT 1200 GCCTCCTGTA CCCGCCCTGG GACAAGAACT TCACCGAGAA TGACCTTCTG GTGCGCATTG 1260 GCAAGCACTC CCGCACCAGG TACGAGCGAA ACATTGAAAA GATATCCATG TTGGAAAAGA 1320 TCTACATCCA CCCCAGGTAC AACTGGCGGG AGAACCTGGA CCGGGACATT GCCCTGATGA 1380 AGCTGAAGAA GCCTGTTGCC TTCAGTGACT ACATTCACCC TGTGTGTCTG CCCGACAGGG 1440 AGACGGCAGC CAGCTTGCTC CAGGCTGGAT ACACGGGGCG GGTGACAGGC TGGGGCAACC 1500 TGAAGGAGAC GTGGACAGCC AACGTTGGTA AGGGGCAGCC CAGTGTCCTG CAGGTGGTGA 1560 ACCTGCCCAT TGTGGAGCGG CCGGTCTGCA AGGACTCCAC CCGGATCCGC ATCACTGACA 1620 ACATGTTCTG TGCTGGTTAC AAGCCTGATG AAGGGAAACG AGGGGATGCC TGTGAAGGTG 1680 ACAGTGGGGG ACCCTTTGTC ATGAAGAGCC CCTTTAACAA CCGCTGGTAT CAAATGGGCA 1740 TCGTCTCATG GGGTGAAGGC TGTGACCGGG ATGGGAAATA TGGCTTCTAC ACACATGTGT 1800 TCCGCCTGAA GAAGTGGATA CAGAAGGTCA TTGATCAGTT TGGAGAGTAG GGGGCCACTC 1860 ATATTCTGGG CTCCTGGAAC CAATCCCGTG AAAGAATTAT TTTTGTGTTT CTAAAACTAT 1920 GGTTCCCAAT AAAAGTGACT CTCAGCG 1947 (2) INFORMATION FOR SEQ ID NO:25: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 2462 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION:SEQ ID NO:25: TCAACAGGCA GGGGCAGCAC TGCAGAGATT TCATCATGGT CTCCCAGGCC CTCAGGCTCC 60 TCTGCCTTCT GCTTGGGCTT CAGGGCTGCC TGGCTGCAGG CGGGGTCGCT AAGGCCTCAG 120 GAGGAGAAAAC ACGGGACATG CCGTGGAAGC CGGGGCCTCA CAGAGTCTTC GTAACCCAGG 180 AGGAAGCCCA CGGCGTCCTG CACCGGCGCC GGCGCGCCAA CGCGTTCCTG GAGGAGCTGC 240 GGCCGGGCTC CCTGGAGAGG GAGTGCAAGG AGGAGCAGTG CTCCTTCGAG GAGGCCCGGG 300 AGATCTTCAA GGACGCGGAG AGGACGAAGC TGTTCTGGAT TTCTTACAGT GATGGGGACC 360 AGTGTGCCTC AAGTCCATGC CAGAATGGGG GCTCCTGCAA GGACCAGCTC CAGTCCTATA 420 TCTGCTTCTG CCTCCCTGCC TTCGAGGGCC GGAACTGTGA GACGCACAAG GATGACCAGC 480 TGATCTGTGT GAACGAGAAC GGCGGCTGTG AGCAGTACTG CAGTGACCAC ACGGGCACCA 540 AGCGCTCCTG TCGGTGCCAC GAGGGGTACT CTCTGCTGGC AGACGGGGTG TCCTGCACAC 600 CCACAGTTGA ATATCCATGT GGAAAAATAC CTATTCTAGA AAAAAGAAAT GCCAGCAAAC 660 CCCAAGGCCG AATTGTGGGG GGCAAGGTGT GCCCCAAAGG GGAGTGTCCA TGGCAGGTCC 720 TGTTGTTGGT GAATGGAGCT CAGTTGTGTG GGGGGACCCT GATCAACACC ATCTGGGTGG 780 TCTCCGCGGC CCACTGTTTC GACAAAATCA AGAACTGGAG GAACCTGATC GCGGTGCTGG 840 GCGAGCACGA CCTCAGCGAG CACGACGGGG ATGAGCAGAG CCGGCGGGTG GCGCAGGTCA 900 TCATCCCCAG CACGTACGTC CCGGGCACCA CCAACCACGA CATCGCGCTG CTCCGCCTGC 960

ACCAGCCCGT GGTCCTCACT GACCATGTGG TGCCCCTCTG CCTGCCCGAA CGGACGTTCT 1020
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AAGCCCCACC TGCACGTGAT CTGCTGGCCC TCAGGCTGCT GCTCTGCCTT CATTGCTGGA 1860
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2462 (2) INFORMATION FOR SEQ ID NO:26: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1437
base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (xi) SEQUENCE
DESCRIPTION:SEQ ID NO:26: ATGCAGCGCG TGAACATGAT CATGGCAGAA TCACCAAGCC
TCATCACCAT CTGCCTTTTA 60 GGATATCTAC TCAGTGCTGA ATGTACAGTT TTTCTTGATC
ATGAAAACGC CAACAAAATT 120 CTGAATCGGC CAAAGAGGTA TAATTCAGGT AAATTGGAAG
AGTTTGTTCA AGGGAACCTT 180 GAGAGAGAAT GTATGGAAGA AAAGTGTAGT TTTGAAGAAC
CACGAGAAGT TTTTGAAAAC 240 ACTGAAAAGA CAACTGAATT TTGGAAGCAG TATGTTGATG
GAGATCAGTG TGAGTCCAAT 300 CCATGTTTAA ATGGCGGCAG TTGCAAGGAT GACATTAATT
CCTATGAATG TTGGTGTCCC 360 TTTGGATTTG AAGGAAAGAA CTGTGAATTA GATGTAACAT
GTAACATTAA GAATGGCAGA 420 TGCGAGCAGT TTTGTAAAAA TAGTGCTGAT AACAAGGTGG
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TCACTCAAGG CACCCAATCA 660 TTTAATGACT TCACTCGGGT TGTTGGTGGA GAAGATGCCA
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GCTCTATCGT TAATGAAAAA 780 TGGATTGTAA CTGCTGCCCA CTGTGTTGAA ACTGGTGTTA
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TAAGTGGCTG GGCAAGAGTC 1080 TTCCACAAAG GGAGATCAGC TTTAGTTCTT CAGTACCTTA
GAGTTCCACT TGTTGACCGA 1140 GCCACATGTC TTCGATCTAC AAAGTTCACC ATCTATAACA
ACATGTTCTG TGCTGGCTTC 1200 CATGAAGGAG GTAGAGATTC ATGTCAAGGA GATAGTGGGG
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AAGAGTGTGC AATGAAAGGC 1320 AAATATGGAA TATATACCAA GGTATCCCGG TATGTCAACT
GGATTAAGGA AAAAACAAAG 1380 CTCACTTAAT GAAAGATGGA TTTCCAAGGT TAATTCATTG
GAATTGAAAA TTAACAG 1437 (2) INFORMATION FOR SEQ ID NO:27: : (i) SEQUENCE
CHARACTERISTICS: (A) LENGTH: 1126 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single
(D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27: GGATTCGAAG GCAAAAACTG
TGAATTATTC ACACGGAAGC TCTGCAGCCT GGACAACGGG 60 GACTGTGACC AGTTCTGCCA
CGAGGAACAG AACTCTGTGG TGTGCTCCTG CGCCCGCGGG 120 TACACCCTGG CTGACAACGG
CAAGGCCTGC ATTCCCACAG GGCCCTACCC CTGTGGGAAA 180 CAGACCCTGG AACGCAGGAA
GAGGTCAGTG GCCCAGGCCA CCAGCAGCAG CGGGGAGGCC 240 CCTGACAGCA TCACATGGAA
GCCATATGAT GCAGCCGACC TGGACCCCAC CGAGAACCCC 300 TTCGACCTGC TTGACTTCAA
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CAAGGACGGG GAGTGTCCCT GGCAGGCCCT GCTCATCAAT 420 GAGGAAAACG AGGGTTTCTG TGGTGGAACC ATTCTGAGCG AGTTCTACAT CCTAACGGCA 480 GCCCACTGTC TCTACCAAGC CAAGAGATTC GAAGGGGACC GGAACACGGA GCAGGAGGAG 540 GGCGGTGAGG CGGTGCACGA GGTGGAGGTG GTCATCAAGC ACAACCGGTT CACAAAGGAG 600 ACCTATGACT TCGACATCGC CGTGCTCCGG CTCAAGACCC CCATCACCTT CCGCATGAAC 660 GTGGCGCCTG CCTGCCTCCC CGAGCGTGAC TGGGCCGAGT CCACGCTGAT GACGCAGAAG 720 ACGGGGATTG TGAGCGGCTT CGGGCGCACC CACGAGAAGG GCCGGCAGTC CACCAGGCTC 780 AAGATGCTGG AGGTGCCCTA CGTGGACCGC AACAGCTGCA AGCTGTCCAG CAGCTTCATC 840 ATCACCCAGA ACATGTTCTG TGCCGGCTAC GACACCAAGC AGGAGGATGC CTGCCAGGGG 900 GACAGCGGGG GCCCGCACGT CACCCGCTTC AAGGACACCT ACTTCGTGAC AGGCATCGTC 960 AGCTGGGGAG AGGGCTGTGC CCGTAAGGGG AAGTACGGGA TCTACACCAA GGTCACCGCC 1020 TTCCTCAAGT GGATCGACAG GTCCATGAAA ACCAGGGGCT TGCCCAAGGC CAAGAGCCAT 1080 GCCCCGGAGG TCATAACGTC CTCTCCATTA AAGTGAGATC CCACTC 1126 (2) INFORMATION FOR SEQ ID NO:28: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 45 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28: GAAGAAGGGA TCCTGGTGCC TCGTGGTTCT GGCACTACAA ATACT 45 (2) INFORMATION FOR SEQ ID NO:29: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 30 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: other nucleic acid (A) DESCRIPTION: /desc = "DNA" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29: CTGGCCTCAA GCTTAACGGA ATTCACCTTT 30 (2) INFORMATION FOR SEQ ID NO:30: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 25 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30: Ala Pro Met Ala Glu Gly Glu Gln Lys Pro Arg Glu Val Val Lys Phe 1 5 10 15 Met Asp Val Tyr Lys Arg Ser Tyr Cys 20 25 (2) INFORMATION FOR SEQ ID NO:31: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 26 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31: Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu Val Val Lys 1 5 10 15 Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys 20 25 (2) INFORMATION FOR SEQ ID NO:32: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 25 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32: Met Ala Ala Gly Ser Ile Thr Thr Leu Pro Ala Leu Pro Glu Gly Gly 1 5 10 15 Asp Gly Gly Ala Phe Ala Pro Gly Cys 20 25

METHODS AND COMPOSITIONS FOR THE SPECIFIC COAGULATION OF VASCULATURE

Claims of corresponding document: **WO 9601653 (A1)**

CLAIMS 1. A binding ligand comprising: (a) a first binding region that binds to a diseased cell, a component of disease-associated vasculature or a component of disease associated stroma; the first binding region operatively linked to (b) a coagulation factor or a second binding region that binds to a coagulation factor.

2. The binding ligand of claim 1, wherein said first binding region comprises an IgG antibody, an IgM antibody or an antigen binding region of an antibody.

3. The binding ligand of claim 2, wherein said first binding region comprises an scFv, Fv, Fab', Fab or F(ab') 2 fragment of an antibody.

4. The binding ligand of claim 2, wherein said first binding region comprises an antigen binding region of an antibody that binds to a tumor cell, a component of tumor vasculature or a component of tumor stroma.

5. The binding ligand of claim 4, wherein said first binding region comprises an antigen binding region of an antibody that binds to a cell surface antigen of a tumor cell .

6. The binding ligand of claim 5, wherein said first binding region comprises an antigen binding region of an antibody that binds to the cell surface tumor antigen p185HER2, milk mucin core protein, TAG-72, Lewis a, carcinoembryonic antigen (CEA) or a tumor-associated antigen that binds to an antibody selected from the group consisting of 9.2.27, OV-TL3, MOv18, B3, KS1/4, 260F9 and D612 .

7. The binding ligand of claim 4, wherein said first binding region comprises an antigen binding region of an antibody that binds to a component of tumor vasculature.

8. The binding ligand of claim 7, wherein said first binding region comprises an antigen binding region of an antibody that binds to a tumor vasculature cell surface receptor - 9. The binding ligand of claim 8, wherein said first binding region comprises an antigen binding region of an antibody that binds to an MHC Class II protein, a VEGF/VPF receptor, an FGF receptor, a TGFP receptor, a TIE, VCAM-1, P-selectin, E-selectin, v3 integrin, pleiotropin, endosialin or endoglin.

10. The binding ligand of claim 9, wherein said first binding region comprises an antigen binding region of an antibody that binds to endoglin.

11. The binding ligand of claim 10, wherein said first binding region comprises an antigen binding region of an antibody that binds to the same epitope as the monoclonal antibody TEC-4 or the monoclonal antibody TEC-11.

12. The binding ligand of claim 9, wherein said first binding region comprises an antigen binding region of an antibody that binds to a VEGF receptor.

13. The binding ligand of claim 12, wherein said first binding region comprises an antigen binding region of an antibody that binds to the same epitope as the monoclonal antibody 3EII, 3E7, 5G6, 4D8 or lOB10.

14. The binding ligand of claim 12, wherein said first binding region comprises an antigen binding region of an antibody that binds to the same epitope as the monoclonal antibody TEC-1l0.

15. The binding ligand of claim 7, wherein said first binding region comprises an antigen binding region of an antibody that binds to a ligand that binds to a tumor vasculature cell surface receptor.

16. The binding ligand of claim 15, wherein said first binding region comprises an antigen binding region of

an antibody that binds to VEGF/VPF, FGF, TGFss, a ligand that binds to a TIE, a tumor-associated fibronectin isoform, scatter factor, hepatocyte growth factor (HGF), platelet factor 4 (PF4), PDGF or TIMP.

17. The binding ligand of claim 16, wherein said first binding region comprises an antigen binding region of an antibody that binds to VEGF/VPF, FGF, TGFP, a ligand that binds to a TIE or a tumor-associated fibronectin isoform.

18. The binding ligand of claim 7, wherein said first binding region comprises an antigen binding region of an antibody that binds to an inducible tumor vasculature component.

19. The binding ligand of claim 18, wherein said first binding region comprises an antigen binding region of an antibody that binds to a tumor vasculature component inducible by a coagulant.

20. The binding ligand of claim 19, wherein said first binding region comprises an antigen binding region of an antibody that binds to a tumor vasculature component inducible by thrombin.

21. The binding ligand of claim 20, wherein said first binding region comprises an antigen binding region of an antibody that binds to P-select in or E-selectin.

22. The binding ligand of claim 18, wherein said first binding region comprises an antigen binding region of an antibody that binds to a tumor vasculature component inducible by a cytokine.

23. The binding ligand of claim 22, wherein said first binding region comprises an antigen binding region of an antibody that binds to a tumor vasculature component inducible by a cytokine released by monocytes, macrophages, mast cells, helper T cells, CD8-positive Tcells or NK cells.

24. The binding ligand of claim 22, wherein said first binding region comprises an antigen binding region of an antibody that binds to a tumor vasculature component inducible by the cytokine IL-1, IL-4, TNF-a, TNF-P or IFN-.

25. The binding ligand of claim 22, wherein said first binding region comprises an antigen binding region of an antibody that binds to E-selectin, VCAM-1, ICAM-1, endoglin or an MHC Class II antigen.

26. The binding ligand of claim 25, wherein said first binding region comprises an antigen binding region of an antibody that binds to E-selectin.

27. The binding ligand of claim 25, wherein said first binding region comprises an antigen binding region of an antibody that binds to an MHC Class II antigen.

28. The binding ligand of claim 7, wherein said first binding region comprises an antigen binding region of an antibody that binds to a ligand:receptor complex but does not bind to the ligand or the factor receptor when the ligand or receptor is not in the ligand:receptor complex.

29. The binding ligand of claim 28, wherein said first binding region comprises an antigen binding region of an antibody that binds to the same epitope as the monoclonal antibody 2E5, 3E5 or 4E5.

30. The binding ligand of claim 4, wherein said first binding region comprises an antigen binding region of an antibody that binds to a component of tumor stroma.

31. The binding ligand of claim 30, wherein said first binding region comprises an antigen binding region of an antibody that binds to tenascin.

32. The binding ligand of claim 30, wherein said first binding region comprises an antigen binding region of an antibody that binds to a basement membrane component.

33. The binding ligand of claim 30, wherein said first binding region comprises an antigen binding region of an antibody that binds to an activated platelet.

34. The binding ligand of claim 30, wherein said first binding region comprises an antigen binding region of an antibody that binds to an inducible tumor stroma component .

35. The binding ligand of claim 34, wherein said first binding region comprises an antigen binding region of an antibody that binds to a tumor stroma component inducible by a coagulant.

36. The binding ligand of claim 35, wherein said first binding region comprises an antigen binding region of an antibody that binds to a tumor stroma component inducible by thrombin.

37. The binding ligand of claim 36, wherein said first binding region comprises an antigen binding region of an antibody that binds to RIBS.

38. The binding ligand of claim 1, wherein said first binding region comprises a ligand or receptor that binds to a diseased cell or to a component of diseaseassociated vasculature.

39. The binding ligand of claim 38, wherein said first binding region comprises a ligand that binds to a tumor cell surface receptor or a soluble binding domain of a receptor that binds to a ligand that binds to a tumor cell surface molecule.

40. The binding ligand of claim 38, wherein said first binding region comprises a ligand or receptor that binds to a component of tumor vasculature.

41. The binding ligand of claim 40, wherein said first binding region comprises a ligand that binds to a tumor vasculature endothelial cell surface receptor.

42. The binding ligand of claim 41, wherein said first binding region comprises VEGF/VPF, FGF, TGFp, a ligand that binds to a TIE, a tumor-associated fibronectin isoform, scatter factor, hepatocyte growth factor (HGF), platelet factor 4 (PF4), PDGF or TIMP.

43. The binding ligand of claim 42, wherein said first binding region comprises VEGF/VPF.

44. The binding ligand of claim 42, wherein said first binding region comprises FGF.

45. The binding ligand of claim 40, wherein said first binding region comprises a soluble binding domain of a receptor that binds to a ligand that binds to a tumor vasculature endothelial cell surface receptor.

46. The binding ligand of claim 45, wherein said first binding region comprises a soluble binding domain of a VEGF/VPF receptor.

47. The binding ligand of claim 1, wherein said first binding region is operatively linked to a coagulation factor.

48. The binding ligand of claim 47, wherein said coagulation factor comprises the vitamin K-dependent coagulant Factor II/IIa, Factor VII/VIIa, Factor IX/IXa or Factor X/Xa.

49. The binding ligand of claim 48, wherein said coagulation factor comprises a vitamin K-dependent coagulation factor lacking the Gla modification.

50. The binding ligand of claim 49, wherein said coagulation factor is prepared by expressing a vitamin Kdependent coagulation factor-encoding gene in a procaryotic host cell.

51. The binding ligand of claim 49, wherein said coagulation factor is prepared by treating the vitamin Kdependent coagulation factor protein to remove or alter the corresponding Glutamic acid residues.

52. The binding ligand of claim 49, wherein said coagulation factor is prepared by preparing an engineered coagulation factor gene that encodes a vitamin Kdependent coagulation factor lacking the corresponding Glutamic acid residues and expressing said engineered gene in a recombinant host cell.

53. The binding ligand of claim 47, wherein said coagulation factor comprises Tissue Factor or a Tissue Factor derivative.

54. The binding ligand of claim 53, wherein said coagulation factor comprises a mutant Tissue Factor

deficient in the ability to activate Factor VII.

55. The binding ligand of claim 54, wherein said coagulation factor comprises a Tissue Factor that includes a mutation in the amino acid region between about position 157 and about position 167.

56. The binding ligand of claim 55, wherein said coagulation factor comprises a mutant Tissue Factor wherein Trp at position 158 is changed to Arg; wherein Ser at position 162 is changed to Ala; wherein Gly at position 164 is changed to Ala; or wherein Trp at position 158 is changed to Arg and Ser at position 162 is changed to Ala.

57. The binding ligand of claim 53, wherein said coagulation factor comprises truncated Tissue Factor.

58. The binding ligand of claim 57, wherein said coagulation factor comprises dimeric truncated Tissue Factor .

59. The binding ligand of claim 47, wherein said coagulation factor comprises Russell's viper venom Factor X activator.

60. The binding ligand of claim 47, wherein said coagulation factor comprises a platelet-activating compound - 61. The binding ligand of claim 60, wherein said coagulation factor comprises thromboxane A2 or thromboxane A2 synthase.

62. The binding ligand of claim 47, wherein said coagulation factor comprises an inhibitor of f ibrinolysis .

63. The binding ligand of claim 62, wherein said coagulation factor comprises a2-antiplasmin.

64. The binding ligand of claim 1, wherein said first binding region is operatively linked to a second binding region that binds to a coagulation factor.

65. The binding ligand of claim 64, further comprising a coagulation factor bound to said second binding region.

66. The binding ligand of claim 64, wherein said second binding region comprises an antigen binding region of an antibody that binds to a coagulation factor.

67. The binding ligand of claim 66, wherein said second binding region comprises an IgG antibody, an IgM antibody, or a scFv, Fv, Fab', Fab or F(ab')2 fragment of an antibody.

68. The binding ligand of claim 66, wherein said second binding region comprises an antigen binding region of an antibody that binds to the vitamin K-dependent coagulant Factor II/IIa, Factor VII/VIIa, Factor IX/IXa or Factor X/Xa .

69. The binding ligand of claim 68, wherein said second binding region comprises an antigen binding region of an antibody that binds to a vitamin K-dependent coagulation factor that lacks the Gla modification.

70. The binding ligand of claim 66, wherein said second binding region comprises an antigen binding region of an antibody that binds to Tissue Factor.

71. The binding ligand of claim 70, wherein said second binding region comprises an antigen binding region of an antibody that binds to a mutant Tissue Factor.

72. The binding ligand of claim 70, wherein said second binding region comprises an antigen binding region of an antibody that binds to. truncated Tissue Factor.

73. The binding ligand of claim 72, wherein said second binding region comprises an antigen binding region of an antibody that binds to dimeric truncated Tissue Factor.

74. The binding ligand of claim 66, wherein said second binding region comprises an antigen binding region of an antibody that binds to Russell's viper venom Factor X activator, thromboxane A2 or a2-.antiplasmin.

75. The binding ligand of claim 1, wherein said first binding region is operatively linked to said coagulation factor or said second binding region via a covalent bond.

76. The binding ligand of claim 75, wherein said first binding region is operatively linked to said coagulation factor or said second binding region via a chemical cross-linker.

77. The binding ligand of claim 75, wherein said binding ligand is a fusion protein prepared by expressing a recombinant vector in a host cell, wherein the vector comprises, in the same reading frame, a DNA segment encoding said first binding region operatively linked to a DNA segment encoding said coagulation factor or second binding region.

78. The binding ligand of claim 1, wherein said first binding region is operatively linked to said coagulation factor or said second binding region using an avidin:biotin combination.

79. The binding ligand of claim 1, further defined as a bispecific antibody comprising a first antigen binding region that binds to a tumor cell, a component of tumorassociated vasculature or a component of tumor-associated stroma, the first antigen binding region operatively linked to a second antigen binding region that binds to a coagulation factor - 80. The binding ligand of claim 79, further defined as an IgG antibody, an IgM antibody or an scFv, Fv, Fab', Fab or F(ab')2 fragment of a bispecific antibody.

81. The binding ligand of claim 79, further defined as a bispecific antibody comprising a first antigen binding region that binds to an MHC Class II protein operatively linked to a second antigen binding region that binds to truncated Tissue Factor.

82. A binding ligand comprising: (a) a first binding region that binds to a tumor cell, a component of tumor-associated vasculature or a component of tumor-associated stroma; the first binding region operatively linked to (b) an engineered coagulation factor or a second binding region that binds to an engineered coagulation factor - 83. The binding ligand of claim 82, wherein said engineered coagulation factor is a vitamin K-dependent coagulant that lacks the Gla modification.

84. The binding ligand of claim 83, wherein said engineered coagulation factor is Factor II/IIa, Factor VII/VIIa, Factor IX/IXa or Factor X/Xa that lacks the Gla modification.

85. The binding ligand of claim 83, wherein said engineered coagulation factor is prepared by expressing a vitamin K-dependent coagulation factor gene in a procaryotic host cell.

86. The binding ligand of claim 83, wherein said engineered coagulation factor is prepared by treating the vitamin K-dependent coagulation factor protein to remove or alter the corresponding Glutamic acid residues.

87. The binding ligand of claim 83, wherein said engineered coagulation factor is prepared by preparing an engineered coagulation factor gene that encodes a vitamin K-dependent coagulation factor lacking the corresponding Glutamic acid residues and expressing said engineered gene in a recombinant host cell.

88. The binding ligand of claim 82, wherein said engineered coagulation factor is a Tissue Factor construct comprising a first Tissue Factor or derivative operatively linked to a second Tissue Factor or derivative.

89. The binding ligand of claim 88, wherein said Tissue Factor construct comprises a truncated Tissue Factor.

90. The binding ligand of claim 89, wherein said Tissue Factor construct comprises a hydrophobic membrane insertion moiety.

91. The binding ligand of claim 90, wherein said Tissue Factor construct comprises a truncated Tissue Factor that includes a stretch of between about 3 and about 20 hydrophobic amino acids.

92. The binding ligand of claim 91, wherein said Tissue Factor construct comprises a truncated Tissue Factor that includes a stretch of hydrophobic amino acids located at the N- or C-terminus of the truncated Tissue Factor.

93. The binding ligand of claim 90, wherein said Tissue Factor construct comprises a truncated Tissue

Factor that includes a fatty acid.

94. The binding ligand of claim 90, wherein said Tissue Factor construct comprises two truncated Tissue Factors, each of which includes a membrane insertion moiety.

95. The binding ligand of claim 88, wherein said Tissue Factor construct comprises a first and second Tissue Factor or derivative operatively linked via a disulfide, thioether or peptide bond.

96. The binding ligand of claim 82, wherein said first binding region is operatively linked to a second binding region that binds to an engineered coagulation factor.

97. The binding ligand of claim 82, wherein said first binding region is operatively linked to an engineered coagulation factor - 98. The binding ligand of claim 97, wherein said first binding region is linked to said engineered coagulation factor via a biologically-releasable bond.

99. The binding ligand of claim 98, wherein said first binding region is linked to said engineered coagulation factor via a selectively-cleavable peptide bond.

100. The binding ligand of claim 99, wherein said first binding region is linked to said engineered coagulation factor via a peptide linker that includes a cleavage site for urokinase, plasmin, Thrombin, Factor IXa, Factor Xa or a metalloproteinase.

101. A Tissue Factor construct comprising a first Tissue Factor or derivative operatively linked to a second Tissue Factor or derivative.

102. The Tissue Factor construct of claim 101, comprising an operatively linked first, second and third Tissue Factor or Tissue Factor derivative.

103. The Tissue Factor construct of claim 102, comprising about five operatively linked Tissue Factors or Tissue Factor derivatives.

104. The Tissue Factor construct of claim 103, comprising about ten operatively linked Tissue Factors or Tissue Factor derivatives.

105. The Tissue Factor construct of claim 104, comprising about twenty operatively linked Tissue Factors or Tissue Factor derivatives.

106. The Tissue Factor construct of claim 101, comprising a truncated Tissue Factor.

107. The Tissue Factor construct of claim 106, comprising a truncated Tissue Factor that includes a hydrophobic membrane insertion moiety.

108. The Tissue Factor construct of claim 107, comprising a truncated Tissue Factor that includes a stretch of between about 3 and about 20 hydrophobic amino acids.

109. The Tissue Factor construct of claim 108, comprising a truncated Tissue Factor that includes a stretch of hydrophobic amino acids located at the N- or C-terminus of the truncated Tissue Factor.

110. The Tissue Factor construct of claim 107, comprising a truncated Tissue Factor that includes a fatty acid.

111. The Tissue Factor construct of claim 107, comprising two truncated Tissue Factors, each of which includes a hydrophobic membrane insertion moiety.

112. The Tissue Factor construct of claim 101, comprising a truncated Tissue Factor modified to contain a terminal cysteine residue.

113. The Tissue Factor construct of claim 112, comprising two truncated Tissue Factors, each of which is modified to contain a terminal cysteine residue.

114. The Tissue Factor construct of claim 112, further comprising an antibody or an antigen binding region of an antibody operatively linked to said cysteine residue.

115. The Tissue Factor construct of claim 106, comprising a truncated Tissue Factor modified to contain a selectively cleavable peptide linker.

116. The Tissue Factor construct of claim 115, wherein said selectively cleavable peptide linker includes a cleavage site for urokinase, plasmin, Thrombin, Factor IXa, Factor Xa or a metalloproteinase.

117. The Tissue Factor construct of claim 116, wherein said selectively cleavable peptide linker includes a cleavage site for an interstitial collagenase, a gelatinase or a stromelysin.

118. The Tissue Factor construct of claim 115, further comprising an antibody or an antigen binding region of an antibody operatively linked to said selectively cleavable peptide linker.

119. The Tissue Factor construct of claim 101, comprising an operatively linked series of units in the sequence: a cysteine residue, a selectively cleavable peptide linker, a stretch of hydrophobic amino acids, a first truncated Tissue Factor and a second truncated Tissue Factor.

120. The Tissue Factor construct of claim 119, comprising an operatively linked series of units in the sequence: a first cysteine residue, a selectively cleavable peptide linker, a first stretch of hydrophobic amino acids, a first truncated Tissue Factor, a second truncated Tissue Factor and a second stretch of hydrophobic amino acids.

121. The Tissue Factor construct of claim 119, further comprising an antibody or an antigen binding region of an antibody operatively linked to said cysteine residue.

122. A pharmaceutical composition comprising, in a pharmacologically acceptable form, a binding ligand that comprises: (a) a first binding region that binds to a diseased cell, a component of disease-associated vasculature or a component of disease associated stroma; the first binding region operatively linked to (b) a coagulation factor or a second binding region that binds to a coagulation factor.

123. The pharmaceutical composition of claim 122, wherein said binding ligand comprises a first binding region covalently linked to a coagulation factor.

124. The pharmaceutical composition of claim 122, wherein said binding ligand comprises a first binding region covalently linked to a second binding region that binds to a coagulation factor.

125. The pharmaceutical composition of claim 124, wherein said binding ligand further comprises a coagulation factor non-covalently bound to said second binding region.

126. The pharmaceutical composition of claim 124, wherein said binding ligand is a bispecific antibody.

127. The pharmaceutical composition of claim 122, wherein said first binding region is operatively linked to Tissue Factor, a Tissue Factor derivative or an antigen binding region of an antibody that binds to Tissue Factor or a Tissue Factor derivative.

128. A kit comprising, in suitable container means: (a) a first pharmaceutical composition comprising a biological agent capable of inducing the expression of a target antigen in disease associated vasculature or disease-associated stroma; and (b) a second pharmaceutical composition comprising a binding ligand that comprises (i) a first binding region that binds to an inducible target antigen of disease associated vasculature or disease associated stroma, the first binding region operatively linked to (ii) a coagulation factor or a second binding region that binds to a coagulation factor.

129. The kit of claim 128, wherein said first pharmaceutical composition comprises a biological agent capable of inducing the expression of a target antigen on a disease-associated vascular endothelial cell.

130. The kit of claim 128, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to an activating antigen on the cell surface of a leukocyte cell and to a tumor antigen on the cell surface of a tumor cell of a vascularized tumor.

131. The kit .of claim 130, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to the activating antigen CD2, CD3, CD14, CD16 (FcR for IgE), CD28 or the T-cell receptor antigen 132. The kit of claim 131, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to CD14 and induces the expression of IL-1 by monocyte/macrophage cells.

133. The kit of claim 132, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to E-selectin.

134. The kit of claim 131, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to CD28 and induces the expression of IFN-y by T-cells.

135. The kit of claim 134, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to an MHC Class II antigen.

136. The kit of claim 134, further comprising a third pharmaceutical composition comprising an agent capable of suppressing MHC Class II antigen expression in the vascular endothelial cells of normal tissues.

137. The kit of claim 136, further comprising a third pharmaceutical composition comprising a cyclosporin.

138. The kit of claim 130, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to the tumor antigen p185HER2, milk mucin core protein, TAG-72, Lewis a, carcinoembryonic antigen (CEA) or a tumor-associated antigen that binds to an antibody selected from the group consisting of 9.2.27, OV-TL3, MOv18, B3, KS1/4, 260F9 and D612.

139. The kit of claim 128, wherein said first pharmaceutical composition comprises a biological agent capable of inducing the expression of a target antigen in a disease-associated stromal component.

140. The kit of claim 139, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to a tumor cell or a component of tumor stroma and to Tissue Factor, a Tissue Factor derivative, prothrombin, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, Factor XI/XIa or Russell's viper venom Factor X activator.

141. The kit of claim 140, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to P select in - 142. The kit of claim 140, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to Eselectin.

143. The use of a binding ligand in the preparation of a medicament for delivering a coagulation factor to disease-associated vasculature, the binding ligand comprising: (a) a first binding region that binds to a diseased cell, a component of disease-associated vasculature or a component of disease associated stroma, the first binding region operatively linked to (b) coagulation factor or a second binding region that binds to a coagulation factor.

144. A use according to claim 143, wherein said binding ligand is used in the preparation of a medicament for delivering an exogenous coagulation factor to diseaseassociated vasculature, the binding ligand comprising a first binding region covalently linked to a coagulation factor 145. A use according to claim 143, wherein said binding ligand is used in the preparation of a medicament for delivering an exogenous coagulation factor to diseaseassociated vasculature, the binding ligand comprising a first binding region covalently linked to a second binding region that is non-covalently bound to a coagulation factor - 146.A use according to claim 143, wherein said binding ligand is used in the preparation of a medicament for delivering an endogenous coagulation factor to diseaseassociated vasculature, the binding ligand comprising a first binding region covalently linked to a second binding region that binds to endogenous coagulation factor and concentrates said factor at said diseaseassociated vasculature upon administration of said medicament to said animal.

147. A use according to claim 143, wherein said medicament is intended for use in treating Benign Prostatic Hyperplasia (BPH), diabetic retinopathy, vascular restenosis, arteriovenous malformations (AVM), hemangioma, neovascular glaucoma, rheumatoid arthritis or psoriasis .

148. A use according to claim 143, wherein said medicament is intended for use in treating an animal with a vascularized tumor.

149. A use according to claim 148, wherein said medicament is intended for use after inducing the expression of a targetable component in tumor-associated vasculature or tumor-associated stroma of an animal, wherein said medicament comprises a binding ligand that comprises a first binding region that binds to the induced targetable component.

150. A use according to claim 149, wherein the expression of the targetable component in tumor-associated vasculature is induced by a cytokine.

151. A use according to claim 150, wherein the expression of the targetable component in tumor-associated vasculature is induced by a cytokine released by leukocyte cells of the animal.

152. A use according to claim 151, wherein the expression of the targetable component in tumor-associated vasculature is induced by a cytokine released by monocytes, macrophages, mast cells, helper T cells, CD8positive T-cells or NK cells of the animal.

153. A use according to claim 151, wherein the expression of the targetable component in tumor-associated vasculature is induced by the cytokine IL-1, IL-4, TNF-a, TNF-P or IFN-Y- 154. A use according to claim 151, wherein the leukocyte cells of the animal are activated to release the cytokine by administering to the animal a pharmaceutical composition comprising an activating antibody that binds to a leukocyte cell surface activating antigen.

155. A use according to claim 154, wherein the activating antibody binds to the leukocyte cell surface activating antigen CD2, CD3, CD14, CD16, FcR for IgE, CD28 or the Tcell receptor antigen.

156. A use according to claim 154, wherein the activating antibody is a bispecific antibody that binds to and cross-links activated leukocytes and tumor cells within the animals' tumor.

157. A use according to claim 156, wherein the activating antibody is a bispecific antibody that binds to CD14 or CD28, and to a cell surface antigen of a tumor cell.

158. A use according to claim 150, wherein the expression of E-selectin, VCAM-1, ICAM-1, endoglin or an MHC Class II antigen is induced in tumor-associated vasculature.

159. A use according to claim 158, wherein the expression of E-selectin is induced in tumor-associated vasculature.

160. A use according to claim 158, wherein the expression of an MHC Class II antigen is induced in tumor-associated vasculature.

161. A use according to claim 160, wherein the expression of MHC Class II antigens by endothelial cells in the animal's normal tissues is suppressed by administering cyclosporin to the animal; and MHC Class II antigen expression in tumor-associated vasculature is subsequently induced by administering to the animal a bispecific antibody that binds to CD28 and to a cell surface antigen of a tumor cell.

162. A use according to claim 160, wherein the expression of MHC Class II antigens by endothelial cells in the animal's normal tissues is suppressed by administering to the animal an anti-CD4 antibody that suppresses IFN-e production in the T cells of the animal; and MHC Class II antigen expression in tumor-associated vasculature is subsequently induced by administering to the animal an IFN-y-producing T cell clone that binds to an antigen in the disease site.

163. A use according to claim 162, wherein the IFN-y- producing T cell clone is prepared by a method that includes the steps of: (a) removing a tissue section from the disease site of the animal; (b) extracting infiltrating leukocytes from the disease site; and (c) expanding the infiltrating leukocytes in vitro to provide the IFN-y producing clone.

164. A use according to claim 149, wherein the expression of the targetable component in tumor-associated vasculature is induced by thrombin.

165. A use according to claim 164, wherein the production of thrombin is induced by administering to the animal a pharmaceutical composition comprising a bispecific antibody that binds to a tumor cell or a component of tumor stroma and to Tissue Factor, a Tissue Factor derivative, prothrombin, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, Factor XI/XIa or Russell's viper venom Factor X activator.

166. A use according to claim 164, wherein the expression of P-selectin is induced in tumor-associated vasculature.

167. A use according to claim 164, wherein the expression of E-selectin is induced in tumor-associated vasculature.

168. The use of a coagulative binding ligand in the preparation of a medicament for treating cancer, the binding ligand comprising: (a) a first binding region that binds to a tumor cell, a component of tumor-associated vasculature or a component of tumor-associated stroma, the first binding region operatively linked to (b) a coagulation factor or a second binding region that binds to a coagulation factor.

169. A use according to claim 168, wherein said medicament is formulated for parenteral administration.

170. A use according to claim 168, wherein said medicament is formulated for injection into a vascularized tumor site.

171. A use according to claim 168, wherein said medicament is suitable for use in a human patient.

172. A use according to claim 168, wherein said coagulative binding ligand comprises a first binding region operatively linked to Tissue Factor, truncated Tissue Factor, dimeric Tissue Factor or an antigen binding region of an antibody that binds to Tissue Factor or a Tissue Factor derivative.
